UNIVERSITAT POLITÈCNICA DE CATALUNYA BARCELONATECH

Endurance races effects in non-elite runners by clinical biomarkers and bioimpedance measurements

Doctoral thesis by compendium of publications

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UNIVERSITAT POLITÈCNICA DE CATALUNYA
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Give every individual the right amount of exercise, not too little and not too much.

Hippocrates P

Al meu marit David

Als meus fills Irina, Martí i Mariona

Agraïments

Aquesta tesi ha estat com una de les curses d'ultrafons de les que faig des de fa anys. Curses en les que has de córrer durant més de vint-i-quatre hores sense parar, curses en les que deus l'èxit d'acabar a l'equip que tens al darrera: família, metge, entrenador, fisioterapeuta i dietista. A l'igual que en una ultramarató, aquesta tesi ha comptat amb un equip humà al darrera imprescindible perquè es pogués dur a terme i arribés al seu termini després de set anys de camí. Val a dir que compaginar la tesi amb ser mare de tres fills, realitzar més de cinc-centes curses arreu del món com a ultrafondista d'elit, escriure tres llibres i co-fundar diverses empreses relacionades amb la salut, l'alimentació i l'esport no ha estat fàcil, però qui va dir que ho seria?

Moltes han estat les persones que han contribuït a que avui aquesta tesi estigui acabada. Segurament em deixaré de citar moltes, però totes elles estan d'alguna manera presents en aquestes pàgines. I entre tots, aquestes paraules per a aquells que hi han destacat d'una manera més especial:

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Emma Roca Rodríguez, octubre 2019.

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Esta tesis ha sido como una de esas carreras de ultrafondo de las que hago desde hace años. Carreras en las que tienes que correr durante más de venticuatro horas sin parar, carreras en las que debes el éxito de terminar al equipo que tienes detrás: familia, médico, entrenador, fisioterapeuta y dietista. Al igual que en una ultramaratón, esta tesis ha contado con un equipo humano detrás imprescindible para que se pudiera llevar a cabo y llegara a su término después de siete años de camino. Cabe decir que compaginar la tesis con ser madre de tres hijos, realizar más de quinientas carreras por todo el mundo como ultrafondista de élite, escribió tres libros y cofundar varias empresas relacionadas con la salud, la alimentación y la deporte no ha sido fácil, ¿pero quién dijo que lo sería?

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Al Dr. Daniel Brotons, gran médico de los deportistas de élite del Consell Català de l'Esport y corredor de maratones, que me lleva como ultrafondista desde hace más de 25 años. Dani ha significado para este proyecto un pilar imprescindible.

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Gracias, muchas gracias a todos, por compartir conmigo esta tesis. Cierro una etapa que comenzó a gestarse en 2012. He aprendido mucho, como científica y como persona, pero el camino sigue porque todavía hay mucho que aportar y la vida ... ¡es un auténtico non-stop!

Emma Roca Rodríguez, octubre 2019.

Abstract

Participation in marathons and ultra-endurance races is growing every day, but it is worrisome that these types of races are becoming mass events, where many runners are not sufficiently prepared. For example, in the 2017 Barcelona marathon there were 19.740 runners, whereas in the 2007 marathon there were 7.430 runners (Zurich Barcelona Marathon). In the age of social media, everyone wants to put his/her photo and comment on Instagram, Facebook, Twitter, etc., about the achievement reached, without caring if his/her health has been compromised. A marathon or longer distance races, require significant exertions and result in an impact on our bodies and bring short and long-term consequences for our health. Never before has running been so fashionable, but to what extent is it done in a healthy manner?

The motivation and merit of this thesis lie in the fact of finding the impact of a 42km run on our body and evaluate the risk of muscle and cardiac damage, inflammation, immunological changes, infection, and renal impairment.

A total of 234 non-elite runners were studied for 4 years (from 2016 to 2019). The articles presented in this thesis are based on the runners who participated in the marathon of 2016. In the article “The Dynamics of Cardiovascular Biomarkers in non-Elite Marathon Runners”, 79 runners were analyzed, in the “Salivary immunity and lower respiratory tract infections in non-elite marathon runners”, 47 runners were analyzed and in the “Effects of a polysaccharide-based multi-ingredient supplement on salivary immunity in non-elite marathon runners”, 41 runners were analyzed.

The changes evaluated in pre-race conditions (baseline), in the end, and 48 h post-race were through blood and saliva biomarkers. Additionally, other variables were assessed before the race, like the nutrition balance and some specific supplement intake, and it was proved how the body weight, recovery time, and amount of training done can directly affect the runner’s performance and health.

The highest elevations in biomarkers associated with muscle damage (CK), inflammation (CRP), and cardiovascular health (Hs-TnT, NT-proBNP, ST2) were seen in the runners with the worse performance time and less training hours pre-race.

Also, the marathon provoked changes in salivary immunity and increased the risk of developing a respiratory tract infection. In addition, supplementation actions like polysaccharide-based ones, positively affected the immune response initiated during a 42 km effort.

By BIVA methodology, a correlation can be made with a displacement of the vector impedance at 24 h and 48 h post-race on the tolerance ellipses of a European Caucasian reference population with biomarkers of renal damage. Transient values of acute kidney injury (AKI) stage 1, more related to inflammatory factors rather than muscle damage, were found in many marathoners which recovered mostly 48 h post-race.

Regarding diet, a correct mono- and poly-unsaturated fatty acid, potassium, and magnesium intake before the marathon influenced better performance and better cardiovascular health.

But, what are the negative long-term effects of these acute changes in clinical biomarkers and bioimpedance measurements after 42 km? How does it affect an inadequate diet and preparation?

This thesis is based on five original publications, three accepted and two under review, separated into three chapters ordered from 2 to 4, starting with cardiovascular biomarkers, immunity, and supplements. In Appendix 2, renal function and bioimpedance vector displacement, and in Appendix 3, diet effects on performance and cardiac health. An introduction in Chapter 1, describes the rationale of the published work and its place within the whole topic of this thesis. As an elite runner for many years and already a veteran, this thesis is my "grain of sand" in the fascinating world of our body’s metabolic responses while running.

Resum

La participació en maratons i curses d'ultra resistència creix cada dia, però és preocupant que aquest tipus de curses es converteixin en esdeveniments massius, on molts corredors no estan prou preparats. Per exemple, a la marató Barcelona del 2017 hi havia 19.740 corredors, mentre que a la marató del 2007 hi havia 7.430 corredors (Zurich Barcelona Marathon). En l'era de les xarxes socials, tots volen posar la seva foto i comentar a Instagram, Facebook, Twitter, etc., sobre l'assoliment aconseguit, independentment de si la seva salut s'ha vist compromesa. Una marató o cursa de llarga distància, requereix esforços significatius i té un impacte en el nostre cos amb unes conseqüències a curt i llarg termini per a la nostra salut. Mai abans havia estat tan de moda córrer, però fins a quin punt es fa de manera saludable?

La motivació i el mèrit d'aquesta tesi rau en el fet de veure l'impacte d'una carrera de 42 km en el nostre cos i avaluar el risc de dany muscular, dany cardíac, d'inflamació, de canvis immunològics, d'infecció, i d'insuficiència renal.

Un total de 234 corredors no elit es van estudiar durant 4 anys (del 2016 al 2019). Els articles presentats en aquesta tesi es basen en els corredors que van participar en la marató del 2016. En l'article "The Dynamics of Cardiovascular Biomarkers in non-Elite Marathon Runners" es van analitzar 79 corredors, en el de "Salivary immunity and lower respiratory tract infections in non-elite marathon runners" es van analitzar 47 corredors i en el de "Effects of a polysaccharide-based multi-ingredient supplement on salivary immunity in non-elite marathon runners" es van analitzar 41 corredors.

Els canvis avaluats en les condicions prèvies a la cursa (estat basal), al final i 48 hores després de la cursa, es van realitzar a través de biomarcadors en sang i saliva. A més a més, es van avaluar altres variables abans de la cursa, com l'equilibri nutricional i una suplementació específica, i es va demostrar com el pes corporal, el temps de recuperació i la quantitat d'entrenament realitzat poden afectar directament el rendiment i la salut del corredor.

Les elevacions més altes en els biomarcadors associats amb el dany muscular (CK), la inflamació (CRP) i la salut cardiovascular (Hs-TnT, NT-proBNP, ST2) es van

observar en els corredors amb pitjor rendiment en cursa i amb menys hores d'entrenament abans de la mateixa.

A més, la marató va provocar canvis en la immunitat salival i va augmentar el risc de desenvolupar una infecció del tracte respiratori. Així mateix, les accions de suplementació com les basades en polisacàrids van afectar positivament la resposta immune iniciada durant l'esforç de córrer 42 km.

Segons el mètode BIVA, es pot fer una correlació del desplaçament del vector impedància a les 24 h i 48 h després de la carrera sobre les el·lipses de tolerància d'una població de referència caucàsica europea, amb els biomarcadors de dany renal. Els valors transitoris de la lesió renal aguda (AKI) tipus 1, més relacionats amb factors inflamatoris que amb el dany muscular, es van trobar en molts maratonians que van recuperar a les 48 h després de la cursa.

Pel que fa a la dieta, una correcta ingesta d'àcids grassos monoinsaturats i poliinsaturats, potassi i magnesi abans de la marató va influir en un millor rendiment i una millor salut cardiovascular.

Però quins són els efectes negatius a llarg termini d'aquests canvis aguts en els biomarcadors clínics i en les mesures de bioimpedància després de 42 km? Com afecta una dieta i una preparació inadequades?

Aquesta tesi es basa en cinc publicacions originals, 3 acceptades i dos en revisió, separades en tres capítols ordenats del 2 al 4, començant amb biomarcadors cardiovasculars, infecció, immunitat i suplementació. En l'Annex 2, la funció renal i el desplaçament del vector de bioimpedància, i en l'Annex 3, els efectes de la dieta sobre el rendiment i la salut cardíaca. Una introducció al Capítol 1 descriu la justificació del treball publicat i el seu lloc dins el tema general d'aquesta tesi. Com a corredora d'elit durant molts anys i ja veterana, aquesta tesi és el meu "gra de sorra" en el fascinant món de les respostes metabòliques que fa el nostre cos mentre correm.

Resumen

La participación en maratones y carreras de ultra resistencia crece cada día, pero es preocupante que este tipo de carreras se conviertan en eventos masivos, donde muchos corredores no están lo suficientemente preparados. Por ejemplo, en la maratón Barcelona de 2017 había 19.740 corredores, mientras que en la maratón de 2007 había 7.430 corredores (Zurich Barcelona Marathon). En la era de las redes sociales, todos quieren subir su foto y comentar en Instagram, Facebook, Twitter, etc., sobre el logro alcanzado, sin importar si su salud se ha visto comprometida. Una maratón o carrera de larga distancia, requiere esfuerzos significativos y tiene un impacto en nuestro cuerpo con unas consecuencias a corto y largo plazo para nuestra salud. Nunca antes había estado tan de moda correr, pero ¿hasta qué punto se hace de manera saludable?

La motivación y el mérito de esta tesis radica en el hecho de ver el impacto de una carrera de 42 km en nuestro cuerpo y evaluar el riesgo de daño muscular, daño cardíaco, inflamación, cambios immunológicos, infección, e insuficiencia renal.

Un total de 234 corredores no elite se estudiaron durante 4 años (del 2016 al 2019). Los artículos presentados en esta tesis se basan en los corredores que participaron en la maratón del 2016. En el artículo “The Dynamics of Cardiovascular Biomarkers in non-Elite Marathon Runners” se analizaron 79 corredores, en el de “Salivary immunity and lower respiratory tract infections in non-elite marathon runners” se analizaron 47 corredores y en el de “Effects of a polysaccharide-based multi-ingredient supplement on salivary immunity in non-elite marathon runners” se analizaron 41 corredores.

Los cambios evaluados en las condiciones previas a la carrera (estado basal), al final y 48 horas después de la carrera, se realizaron a través de biomarcadores de sangre y saliva. Además, se evaluaron otras variables antes de la carrera, como el equilibrio nutricional y una suplementación específica, y se demostró como el peso corporal, el tiempo de recuperación y la cantidad de entrenamiento realizado pueden afectar directamente el rendimiento y la salud del corredor.

Las elevaciones más altas en los biomarcadores asociados con el daño muscular (CK), la inflamación (CRP) y la salud cardiovascular (Hs-TnT, NT-proBNP, ST2) se

observaron en los corredores con peor rendimiento en carrera y con menos horas de entrenamiento antes de la misma.

Además, la maratón provocó cambios en la inmunidad salival y aumentó el riesgo de desarrollar una infección del tracto respiratorio. A su vez, las acciones de suplementación como las basadas en polisacáridos afectaron positivamente la respuesta inmune iniciada durante el esfuerzo de correr 42 km.

Según el método BIVA, se puede hacer una correlación del desplazamiento del vector impedancia a las 24 h y 48 h después de la carrera sobre las elipses de tolerancia de una población de referencia caucásica europea, con los biomarcadores de daño renal. Los valores transitorios de la lesión renal aguda (AKI) tipo 1, más relacionados con factores inflamatorios que con el daño muscular, se encontraron en muchos maratonianos que recuperaron a las 48 h después de la carrera.

Con respecto a la dieta, una correcta ingesta de ácidos grasos monoinsaturados y poliinsaturados, potasio y magnesio antes de la maratón influyó en un mejor rendimiento y una mejor salud cardiovascular.

¿Pero cuáles son los efectos negativos a largo plazo de estos cambios agudos en los biomarcadores clínicos y las mediciones de bioimpedancia después de 42 km? ¿Cómo afecta una dieta y una preparación inadecuadas?

Esta tesis se basa en cinco publicaciones originales, tres aceptadas y dos en revisión, separadas en tres capítulos ordenados del 2 al 4, comenzando con biomarcadores cardiovasculares, infección, inmunidad y suplementación. En el Anexo 2, la función renal y el desplazamiento del vector de bioimpedancia, y en el Anexo 3, los efectos de la dieta sobre el rendimiento y la salud cardíaca. Una introducción en el Capítulo 1 describe la justificación del trabajo publicado y su lugar dentro del tema general de esta tesis. Como corredora de élite durante muchos años y ya veterana, esta tesis es mi "grano de arena" en el fascinante mundo de las respuestas metabólicas que hace nuestro cuerpo mientras corremos.

Table of contents

Agraïments **i**

Agradecimientos **iii**

Abstract **v**

Resum **vii**

Resumen **ix**

List of Figures and Tables **xv**

Abbreviations **xix**

Preface **xxi**

Justification **xxiii**

Chapter 1: Introduction **1**

1. Endurance exercise and electrical bioimpedance measurement **5**

1.1 Bioimpedance basics and biological variables **5**

1.2 Bioimpedance measurements **9**

1.2.1 Whole-body bioimpedance **9**

1.2.2 Localized bioimpedance **11**

1.3 Bioimpedance measurement interpretation **12**

1.3.1 Conventional bioelectrical impedance methods **12**

1.3.2 Bioelectrical Impedance Vector Analysis (BIVA) **13**

2. Endurance exercise and skeletal muscle **15**

2.1 Muscle tissue **15**

2.1.1 Muscle training **19**

2.1.2 Muscle fatigue **24**

2.1.3 Muscle damage and injury **25**

2.2 Muscle blood biomarkers **28**

2.2.1 Muscle damage biomarkers **28**

2.2.2 Inflammation biomarkers **31**

2.3 L-BIA measurements **34**

3. Endurance exercise and cardiovascular health **35**

3.1 Cardiac muscle **37**

3.2 Athlete's heart **38**

3.3 Sudden cardiac death in athletes **40**

3.4 Cardiac blood biomarkers **45**

3.4.1 High-sensitivity troponin T (Hs-TnT) **47**

3.4.2 Amino-terminal pro-B type natriuretic peptide (NT-proBNP) **49**

3.4.3 Suppression of tumorigenicity 2 (ST2) **50**

4. Endurance exercise and immunity **52**

4.1 Salivary immunity **57**

4.2 Saliva biomarkers **58**

4.2.1 Antimicrobial peptides: sIgA, lysozyme and lactoferrin **58**

4.2.2 Chemokines: Groα, Groβ, MCP-1 **60**

4.2.3 Anti-inflammatory proteins:Angiogenin, Acrp, Siglec 5 **61**

4.3 Glycans **62**

5. Endurance exercise and nutrition **65**

5.1 Energy needs **66**

5.2 Macronutrients **67**

5.2.1 Carbohydrates **69**

5.2.2 Fats **70**

5.2.3 Proteins **73**

5.3 Micronutrients **74**

5.4 Hydration **76**

5.5. Nutrition and hydration status biomarkers **80**

5.6. Whole-body bioimpedance measurements **81**

6. Hypothesis **83**

7. Objectives **85**

Chapter 2: The dynamics of cardiovascular biomarkers in non-elite marathon runners **87**

Chapter 3: Salivary immunity and lower respiratory tract infections in non-elite marathon runners **93**

Chapter 4: Effects of a polysaccharide-based multi-ingredient supplement on Salivary Immunity in non-Elite Marathon runners **107**

Chapter 5: Conclusions **117**

Chapter 6: Perspectives **121**

Bibliography **123**

Appendix 1: Publications derived from this thesis **137**

Appendix 2: Relationship Between Bioimpedance Vector Displacement and Renal Function After a Marathon in Non-Elite Runners (505424) **139**

Appendix 3: Macronutrient and mineral intake effect on racing time and cardiovascular health in non-elite marathon runners (NUT-D-19-01000) **141**

Appendix 4: Publications derived from data acquired before this thesis **143**

Appendix 5: Races or events analyzed 2012-2019 **147**

Appendix 6: Races done as an ultra-endurance athlete **149**

List of Figures

Figure 1. Outline of some of the physiological responses to exercise. Retrieved from Hawley et al. (2014). **2**

Figure 2. Benefits of regular endurance exercise. Adapted from Rowe et al. (2014). **3**

Figure 3. Electric model of a cell. R_E extracellular resistance, C_M capacitive influence of cell membranes, R_I intracellular resistance. Retrieved from Lukaski (1996). **6**

Figure 4. (a): Geometric relationships among the resistance (R), reactance (Xc, as CM capacitance), impedance (Z), and phase angle with the respective formulas. (b): Representation of the body as a parallel resistor-capacitor (RC) equivalent circuit. Retrieved and adapted from Lukaski et al. (2019). **7**

Figure 5. Dispersion regions for biologic tissue proposed by Schwan (Schwan, 1957). **8**

Figure 6. (a) In body composition analysis, the semi-circle of Cole's model for cell suspension is utilized (b) The curve formed by Z vectors on the R-Xc plane (impedance locus) is a semicircle with a depressed center; smaller versus larger circles correspond to a more versus less hydrated status as progressive tissue dehydration increases tissue impedance. (R: resistance; R0: resistance at 0 frequency; $R\infty$: at infinity; Xc: reactance). Retrieved from Piccoli et al. (2005a) **9**

Figure 7. Electrode configuration, injectors and detectors (I,V), for standard measurement or whole-body. **10**

Figure 8. Location of the L-BIA electrodes in (a) twin muscle in Menorca Ultra Trail and (b) hamstring muscle in the Barcelona Marathon. **11**

Figure 9. BIVA pattern (RXc graph). The three tolerance ellipses represent the 50th, 75th and 95th percentiles of the impedance vector bivariate normal distribution of a reference population of healthy caucasian males. Retrieved from Piccoli et al. (1994, 2002c). **14**

Figure 10. Muscle fiber organization. Retrieved from OpenStax, Anatomy & Physiology: Support and Movement (2014). **16**

Figure 11. (A) Sarcomere structure with actin and myosin units as main proteins. (B) Contraction sequence. Retrieved from OpenStax, Anatomy & Physiology: Support and Movement (2014). **17**

Figure 12. Three mechanisms by which ATP can be regenerated: creatine phosphate metabolism (a), anaerobic glycolysis (b) and aerobic respiration (c). Retrieved from OpenStax, Anatomy & Physiology: Support and Movement (2014). **18**

Figure 13. Outline of the muscle fiber types ranging from fast and glycolytic fibers to slow and mostly oxidative fibers. Retrieved from Rowe et al. (2014). **20**

Figure 14. Pathways related to glucose, lactate, and fatty acid metabolism in fast (red arrows) or slow (green arrows) muscle fibers. Retrieved from Schiaffino and Reggiani (2011). **22**

Figure 15. Theoretical framework summarizing possible mechanisms for delayed muscle soreness following eccentric exercise. Retrieved from Hyldahl and Hubal (2014). **27**

Figure 16. The major pathways related to the CRP production. Retrieved from Vashist et al. (2015). **33**

Figure 17. Drawing of muscle damage due to hamstring strain. **34**

Figure 18. The evolutionary cardiovascular disease pyramid. The way to prevent initial or recurrent cardiac events is to modify unhealthy lifestyle habits that are the base of the pyramid (CHF: congestive heart failure; MI: myocardial infarction; PAD: peripheral arterial disease). Retrieved from Franklin (2014). **36**

Figure 19. Cardiac muscle and its intercalated discs with gap junctions and desmosomes. Retrieved from OpenStax, Anatomy & Physiology: Cardiac Muscle Tissue (2014). **37**

Figure 20. Normal heart vs athlete's heart vs pathological heart. Retrieved from Medical doctor (https://www.picluck.net/media/947934150023816202_1591812152). **39**

Figure 21. Age-dependent changes in incidence and etiology of sudden cardiac death. RV, right ventricular; VT, ventricular tachycardia. Retrieved from La Gerche et al. (2013). **42**

Figure 22. Risk of new-onset atrial fibrillation (AF) in older adults depending on exercise intensity. Retrieved from O'Keefe et al. (2014) and adapted from Mozzafarian et al. (2008). **43**

Figure 23. Total coronary plaque volume (noncalcified plaque volume and calcified plaque volume) in male marathoners compared to sedentary controls. Retrieved from O'Keefe et al. (2014) and adapted from Schwartz et al. (2014). **43**

Figure 24. Causes of cardiac arrest in 59 runners (42±13 years) over 10.9 million analyzed (HCM: hypertrophic cardiomyopathy, PHCM: possible hypertrophic cardiomyopathy). Retrieved from Kim et al. (2012). **44**

Figure 25. Seven axes profile representing biomarkers of each class. The resultant biomarker profile should enhance prevention, treatment and prognostication. Retrieved from Braunwald (2009). **46**

Figure 26. Scheme of the cardiac muscle where the 3 troponin isoforms can be located in relation to actin and tropomyosin. Retrieved from Shave et al. (2010). **47**

Figure 27. The structure of proBNP, BNP, and N-terminal proBNP (BNP=B-type natriuretic peptide; RAAS=renin-angiotensin-aldosterone system). Retrieved from Bhalla et al. (2004). **49**

Figure 28. Cooperation between Innate and Adaptive Immune Responses being more effective. Retrieved from OpenStax, Anatomy & Physiology: The Lymphatic and Immune System (2014). **52**

Figure 29. "J" shaped model suggesting that moderate exercise may lower risk of respiratory infections while very high-intensity exercise may increase the risk. Retrieved from Nieman (1997). **53**

Figure 30. The 'open window' theory. Moderate exercise causes mild immune changes; in contrast, prolonged, marathon-type exercise leads to immune dysfunction that increases the likelihood for opportunistic upper respiratory tract infections. Retrieved from Nieman (2007). **55**

Figure 31. Schematic of potential mediators of inflammatory cell interactions with injured muscle (NO: nitric oxide; μPAR: urokinase type plasminogen-activator receptor; μPA, urokinase; MCP-1: monocyte chemoattractant protein-1; FKN:fractalkine; MDC: macrophage-derived chemokine; VEGF: vascular endothelial growth factor; SOD: superoxide dismutase; MPO:myeloperoxidase). Retrieved from Tidball (2005). **56**

Figure 32. Schematic representation of sIgA (Fab: fragment antibody binding portion, Fc: fragment crystallizable portion). Retrieved from Bishop & Gleeson (2009). **58**

Figure 33. (a) Common classes of animal glycans; (b) schematic representation of a glycoprotein normally embedded in the membrane bilayer (Thy-1) with three N-glycans (blue), a glycosylphosphatidylinositol (GPI-glycan, green), lipid anchor whose acyl chains (yellow), polypeptide (purple). Retrieved from Essentials of Glycobiology (chapter 1), Varki et al. (2009). **62**

Figure 34. Glycans and cellular biology. Complex glycans play critical roles in intercellular and intracellular processes, which are fundamentally important to the development of multicellularity. Retrieved from Hart & Copeland (2010). **63**

Figure 35. Percentage by weight of the different organs of the body (a) and of the body water, fat and CHO stores (b). Adapted from Noakes (2002). **67**

Figure 36. Carbohydrate intake guidelines. CHO intake recommendations increase with increasing duration of exercise and also the CHO type and nutritional training vary. Retrieved from Jeukendrup (2014). **70**

Figure 37. The crossover concept: when exercise intensity increases, there is a decrease in energy derived from fat and an increase in CHO. The crossover point describes when the CHO contribution to substrate oxidation supersedes that of fat. MFO: maximal fat oxidation. Retrieved from Purdom et al. (2018). **71**

Figure 38. Diagram of intra and extracellular electrolyte distribution (a). The sodium-potassium pump powered by ATP to transfer sodium out of the cytoplasm and potassium into the cell (b). Retrieved from Betts et al., Anatomy and Physiology: Fluid, Electrolyte, and Acid-Base Balance (2017). **78**

Figure 39. Possible bidirectional relationship between exercise-associated hyponatremia (EAH) and rhabdomyolysis. Retrieved from Hew-Butler et al. (2017). **80**

Figure 40. Graphic summary of the results obtained in this thesis. **119**

List of Tables

Table 1. Main characteristics of Skeletal and Cardiac muscle. Adapted from Marieb, Human Anatomy & Physiology (2004). **15**

Table 2. MET metabolic equivalent task of some main activities and its equivalent in Kcal/ min for a person with 70 kg weight. mph, miles per hour. Adapted from Ainsworth et al. (1995, 2000). **23**

Table 3. List of the main macronutrients. **68**

Table 4. List of the main micronutrients. **75**

Abbreviations

AA	Advance Ambrotose© complex powder
ACRP	Adipokine or adinopectin
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AF	Atrial fibrillation
AHA	American Heart Association
AKI	Acute kidney injury
AMI	Acute myocardial infarction
AMP	Adenosine monophosphate
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BCAA	Branched chain amino acid
BIA	Bioimpedance
BIS	Bioimpedance spectroscopy
BIVA	Bioelectrical Impedance Vector Analysis
BMI	Body mass index
C	Capacitor
Ca^{+2}	Calcium
CGM	Central governor model
CHO	Carbohydrates
CK	Creatine Kinase
C_M	Capacitive influence of cell membranes
CRP	C-reactive protein
cTnT	Cardiac Troponin T
CV	Coefficients of variation
CVD	Cardiovascular diseases
DAMPs	Damage associated molecular patterns
DHA	Docosahexaenoic acid
DOMS	Delayed onset muscle soreness
EAH	Exercise associated hyponatremia
ECF	Extracellular fluid
ECG	Electrocardiogram
ECW	Extracellular water
EF	Ejection fraction
EFA	Essential fatty acid
EMI	Electrical impedance myography
EPA	Eicosapentaenoic acid
FAox	Fatty acid oxidation
Fc	Fragment crystallizable
FFM	Fat-free mass
FM	Fat mass
HF	Heart Failure
GFR	Glomerular filtration rate
Groα	Growth-regulated oncogene-alpha
Groβ	Growth-regulated oncogene-beta
HDL	High density lipoprotein
Hs-cTnT	High-sensitivity cardiac troponin T
ICW	Intracellular water
IL	Interleukin
K	Potassium
L-BIA	Segmental bioimpedance analysis
Lac	Lactoferrin
LCHF	Low-carbohydrate high-fat diets
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
Lys	Lysozyme
MCP-1	Monocyte chemoattractant protein 1
MET	Metabolic equivalent task
Mg	Magnesium
NT-proBNP	Amino-terminal pro-B type natriuretic peptide
NK	Natural killer
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
PA	Phase angle
PAMPs	Pathogen-associated molecular patterns

Abbreviations

R	Resistance
R_E	Extracellular resistance
R_I	Intracellular resistance
RI	Respiratory infections
RPE	Rating of perceived exertion
SD	Standard deviation
sAMPs	Salivary antimicrobial proteins
sIgA	Salivary Immunoglobulin A
Siglec 5	Sialic acid-binding Ig-like lectin 5
ST2	Suppression of tumorigenicity 2
TBW	Total body water
TLR	Toll-like receptors
TNF	Tumor necrosis factor
VO_2	Oxygen consumption
Xc	Capacitive Reactance
Z	Impedance

Preface

Doing more than 100 races (marathons and ultramarathons) during the last 15 years has given me a very broad vision of what it means to be a runner (Appendix 6). I have seen the importance of good preparation before any challenge, and not only at the physical level, but also at a nutritional and medical level.

Running all this time, suffering injuries, dehydration, muscle damage or injury, infection or immunosuppression, has illustrated to me as a biochemist the lack of published scientific rigor, and the need to give this knowledge to both non-elite and elite runners like myself have accumulated lots of km and races.

Since 2012 I started a research project to explore the impact of running at a muscular, cardiac, physiological, and genetic level. This project denoted SUMMIT (Salut en les Ultra Maratons i els seus LíMITs, health in the ultramarathons and their limits) has analyzed more than 500 runners in more than 25 different races or events (Appendix 5). First I focused on mountain races, both in short and long distances, and then in asphalt marathons like the Zurich Barcelona Marathon, which is the center of this doctoral thesis.

Through the discovery of how a mountain race affects the skeletal and cardiac muscle, gene expression, heart variability, right ventricle adaptation or pulmonary vascular function; I wanted to see what happens in the discipline where there are more fans in the whole world, the asphalt marathon.

Initially, I did not focus on the elite runner, since they are a minority of the total number of practitioners and difficult to convince, and I went to find the amateur runner, enthusiastic and predisposed, with more or less experience in marathons but representing more than 99% of the currently enrolled participants. The main objective was to analyze and determine running risk factors and find the tools to prevent them.

During the first stage of the SUMMIT Project, starting in 2012 with the mountain races, some articles were published (Appendix 4) before enrolling in the thesis at the UPC. The second stage started in 2016 when I enrolled in the UPC doctoral program, Biomedical Engineering, analyzing the Barcelona marathon from which I have already published 3 articles and 2 more are under submission

The races carried out by myself and as a result of all these investigations, provide a knowledge that goes far beyond what can be found by a researcher who does not run, or by someone who performs only laboratory tests.

Justification

This study is born from the interest to deepen knowledge and future prevention tools regarding the impact of running on people, that without being sufficiently prepared, exercise challenges that go beyond healthy.

This thesis can help coaches, sports clinicians, researchers and especially athletes to better understand some physiological changes associated with running, and establish guidelines for better performance, recovery, and general health. But above all, to determine long-term negative effects on acute metabolism changes that occur in each race.

From the SUMMIT project (Health in the UltraMarathons and their limits) started in 2012, the objective was to group the knowledge of different institutions, from Universities and Hospitals, and together deepen a broad knowledge of the limits of a runner. SUMMIT with the Electronic Engineering Department of the Universitat Politècnica de Catalunya, the Germans Trias i Pujol Hospital and the Santa Creu i Sant Pau Hospital have analyzed different blood and saliva variables together with bioimpedance measurements and dietary intake analysis on asphalt marathoners, that are reflected in this doctoral thesis.

The works that make up this doctoral thesis are the first publications in journals indexed in the JCR (Q1 and Q2) related to the impact of a marathon run at different levels.

Chapter 1: Introduction

Someone who decides to run more than 1 h non-stop, must consider some previous tasks like appropriate training of the selected distance, good nutrition and hydration preparation, supplementation if needed, suitable rest, appropriate equipment and above all to be healthy with a good report from a medical check-up. Even if we run and perform correctly, we are not exempt from related dangers, although what can really kill us is a sedentary lifestyle.

Despite the fact that exercise is a fundamental component of the human condition for evading predators and food procurement, we are the only primates capable of running long distances, standing out above other primates (Bramble and Lieberman, 2004). The exercise undertaken to an individual's maximum can be a complex process involving synchronized and integrated activation of multiple tissues and organs at a cellular and systemic level, representing a major challenge to whole-body homeostasis. Muscle contraction, for example, increases metabolic activity and requires fuel and oxygen to maintain itself, activating systemic responses such as cardiovascular, respiratory, neuronal, and hormonal that mitigate homeostatic threats (Figure 1) (Hawley et al., 2014).

The lower limit of physical exercise that brings great health benefits daily is known to be moderate aerobic exercise for a minimum of 30 minutes 5 days a week (Rowe et al., 2014; Subirats et al., 2012), but there is a great lack of knowledge about where the dividing line of the upper limit is, from which sport does not provide a clear benefit (Figure 2) (Hoffman and Krishnan, 2014; O'Keefe et al., 2014). Is an endurance exercise, like a marathon or an ultramarathon, an activity that goes over this limit?

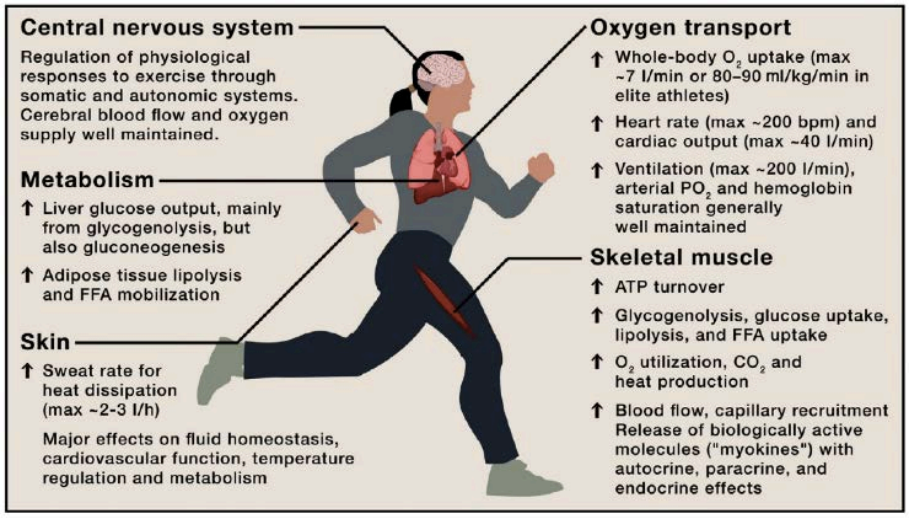


Figure 1. Outline of some physiological responses to exercise. Retrieved from Hawley et al. (2014).

It must be considered that each person has their own response to physical exercise, and for example, the effect that training can have on physiological adaptations is interpersonal (Noakes, 2002). For this reason, each one must be their own reference and act accordingly. A personalized exercise prescription may confer the health benefits of regular training, and we cannot emulate other runners that may have nothing to do with us. There is complex biology behind the diversity in the adaptive response to training between runners. Being able to describe this response through the identification of molecules, metabolic pathways, and even new treatments, the benefit that exercise gives to each person can be maximized (Gabriel and Zierath, 2017). When our preparation nor nutrition is inadequate, numerous homeostatic processes are affected, some key biomarkers can be altered and can limit our health, performance, and recovery. Data from long-term biomarker analysis associated with nutrition, hydration, muscle status, cardiovascular endurance, injury risk, and inflammation can help in the preventative detection of injury or sport negative effects on performance (Lee at al., 2017).

The number of non-elite runners participating in such long races is dramatically increasing every day (Hoffman et al., 2010; IAU Ultra Marathon). Marathon races have become mass events where many runners are not sufficiently prepared for, because the average finishing times of nearly all the most important city marathons increased continuously in the last 15 years (Marathon Guide, 2014).

Either marathon or ultramarathon viewed from the perspective of social and cultural anthropology is a "sacrificial" sport. It supports psychological and physical pain, its growth is unstoppable and goes beyond a commercial, pedagogical, or healthy value. According to sociologists, those who practice this type of sport do so, more as a social ritual that reinforces the identity of oneself and not as a mere healthy physical exercise (Cachan, 2014). Nowadays finishing a race or improve a personal best, rather than besting the opponents, serves a great majority of runners as a precept of sacrifice and becomes a philosophy of life in its most existential sense (Cachan, 2014), but at what price?

Figure 2. Benefits of regular endurance exercise. Adapted from Rowe et al. (2014).

Much of a marathon battle is mental, and often fatigue becomes more bearable the nearer we get to the finish. But normally when we are running a marathon or ultramarathon poorly, being an elite or non-elite athlete, means that we are probably underperforming due to muscle damage. The reason we are running slowly is that our brain is trying to tell us to stop moving, the brain is responding to negative messages from the muscles, which are trying desperately to tell our brain that they are distressed and in need of rest (Noakes, 2002). Our brain is using the symptoms of fatigue as key to a regulator to insure that the exercise is completed before harm develops, but we do not always obey, we want to finish the race at any risk, and there can be consequences.

The biomarkers analyzed in this thesis, both in blood and saliva, are related to performance, recovery, and health in general. These biomarkers evaluate muscle and cardiac damage, inflammation, immunity, infection, renal function, as well as nutritional status, and hydration. In addition, localized and global bioimpedance measurements, have been used as a fast and non-invasive method, to assess different muscle group symmetry, activation, injury, hydration and other clinical settings (Nescolarde et al., 2008, 2015 and 2017).

We must consider that the reference ranges of the biomarkers analyzed are based on the general population and not specifically on runners. Repeated measurements in future studies which in this population type must be done, can help doctors, trainers and athletes to identify more specific changes associated with the risk of injury, overtraining, or diminishing performance.

1. Endurance exercise and electrical bioimpedance measurement

1.1 Bioimpedance basics and biological variables

Bioimpedance (BIA) is a non-invasive, fast, painless, and portable method that deals with a passive electrical property of the tissue: the ability to oppose electric current flow. BIA is an indirect assessment of human body composition, it serves as a support tool in the analysis of changes in hydration and nutrition with global bioimpedance (BIA “whole-body”) (Foster and Lukaski, 1996; Lukaski and Piccoli, 2012); or in the detection of overactivation and muscle injury with localized bioimpedance (L-BIA) (Nescolarde et al., 2013).

BIA, based on the principle that biological tissue behave as conductors to a greater or lesser extent of electric current and/or dielectrics (insulators) depending on their composition can be represented by a model shown in Figure 3. This model includes a resistance attributable to an extracellular path, dependent on the amount and composition of extracellular fluid, and a complex intracellular path, including cell membrane capacitance and resistance. The model indicates three critical biological indexes (intracellular conductor, extracellular conductor, and membrane capacitance) that may be approximated with bioelectrical impedance variables (Lukaski, 1996).

Resistivity (inverse of conductivity) and permittivity (accumulation of charge at interfaces) are two intensive material properties in which frequency values dependency vary among tissues, being the muscle with the highest permittivity and the bone with the highest resistivity (Foster and Lukaski, 1996). For this reason, intra and extracellular electrolyte solutions of all soft tissues (in particular non-fatty tissues) are good conductors and optimally analyzed by BIA, whereas bone is not so easily crossed by the electric currents and behaves like a bad conductor (insulator). In adipose tissue, the current can pass through the electrolyte solutions of the interstitium and the adipocytes, but not by the hydrophobic lipid drops, which do not conduct current (Grimnes and Martinsen, 2000; Piccoli et al., 2002a).

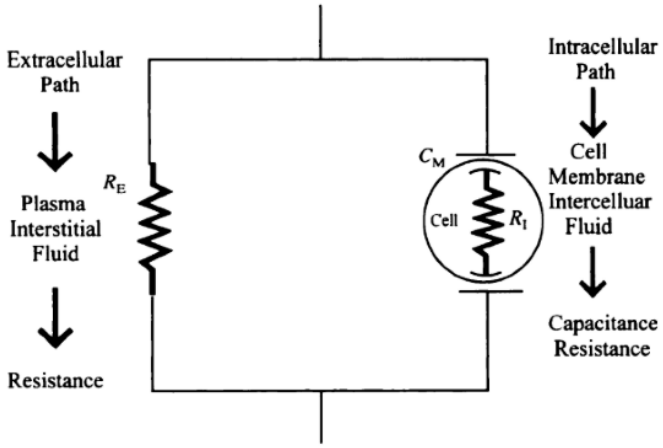


Figure 3. Electric model of a cell. R_E extracellular resistance, C_M capacitive influence of cell membranes, R_I intracellular resistance. Retrieved from Lukaski (1996).

Many diverse models of cell suspension like that depicted in Figure 3, in series and parallel orientations, form the human body and they are characterized by mixtures of physiological fluids and electrolytes acting as resistors, cell membranes, and tissue interfaces acting as capacitors. The term used to describe the opposition to the flow of an alternating current by any biological conductor is the Impedance (Z) (called resistance, R, when direct current is applied). The alternating current flows through resistive and capacitive elements that can be measured with the impedance components:

-The resistance component (R, Ω) or the real part of Z, is frequency independent (it has the same measurement value with either direct or alternating current) opposition to the flow of current, being the inverse of conductance. According to Ohm's Law, R equals the voltage (E) divided by the current (I) transmitted by ions in aqueous solution (R= E/I). Resistance arises from collisions of the current-carrying charged particles through the intra- and extra-cellular electrolyte solutions, indicating extracellular fluid (ECF) and electrolyte composition.

-The reactance (Xc) or the imaginary part of Z, is an additional frequency-dependent opposition to the movement, being the reciprocal of capacitance or the voltage stored by a condenser for a brief period of time. It is determined by the differential properties of the tissues and indicates cell membrane mass and function.

Impedance is described as a complex number ($Z^2 = R^2 + Xc^2$) and characterizes the specific fluid and cellular components of an organism (Lukaski, 1996). Impedance is calculated as the vector with length and position on the bivariate RXc graph and the phase angle (PA) describes the position of Z, evaluating the intra- and extra-cellular fluid (Figure 4a). The phase angle is also the geometric representation of the phase change created when the administered current is delayed behind the voltage due to capacitance causes (Figure 4b) (Lukaski et al., 2019).

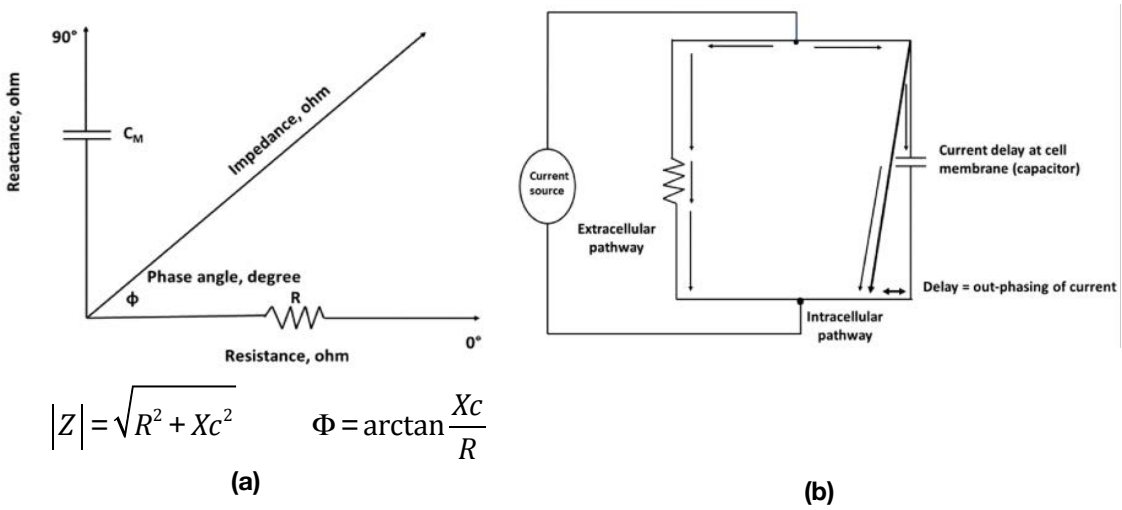


Figure 4. (a): Geometric relationships among the resistance (R), reactance (Xc, as CM capacitance), impedance (Z), and phase angle with the respective formulas. (b): Representation of the body as a parallel resistor-capacitor (RC) equivalent circuit. Retrieved and adapted from Lukaski et al. (2019).

The calculation of fluid volumes and compartments derived from conventional BIA is based on the proportionality between Z and the dimensions of length and area of a conductive cylinder. The extremities and trunk of the human body can be considered as conductors of irregular cylindrical shape, with a length L (m) easily determinable using the hand-foot distance of the person (in the standard BIA height is used as a surrogate for the length of the body), and an area in its cross-section A (m^2) more difficult to determine. By Ohm's law, the impedance Z in a cylindrical conductor is proportional to the length multiplied by a complex resistivity (ρ) that varies among tissues, and inversely proportional to the cross-section, $Z = \rho L / A$. This formula, in general, has prediction errors like the electric-volume model based on the tissues anisotropy (current conduction is not constant in different directions), human body geometry different than a cylinder or the biological variability among subjects with

different body composition and body geometry (Kushner, 1992; Lukaski, 1996; Piccoli et al., 2005).

When a current under 10 GHz is used to go through soft tissues, there are three different frequency intervals due to dispersion phenomena and dielectric relaxation (Schwan, 1957). Each of these frequency zones has a concrete name: alfa (related with low frequencies, Hz) beta (related with middle frequencies, kHz) and gamma (related with high frequencies, GHz) (Figure 5). At high frequencies the current penetrates cells, whereas at low ones the current moves around cells. Multi-frequency measurements can provide a precise index of extracellular water (ECW), total body water (TBW) and free fat mass (FFM), but is very complex, has high instability, low repeatability (there are a lot of inter-individual differences in the distribution of fluids) and is still under investigation. On the other hand, mono-frequency with 50 kHz measurement that has a maximum Xc and Pa value among the 1 kHz-1 MHz range, it is validated by bioimpedance spectroscopy (BIS) following the Cole's model approach (Figure 6) and predominantly intervene polarization and relaxation phenomena in the interfaces, especially in the cell membrane. For this reason, in body tissues with a clinical objective, 50 kHz is the optimum frequency to use in the alfa frequency zone far below the thresholds of human body perception (Piccoli et al., 2005). Also at 50 kHz the measurement error (as coefficient of variation) of the standard mono frequency technique is small, around 2% (Piccoli et al., 2002b).

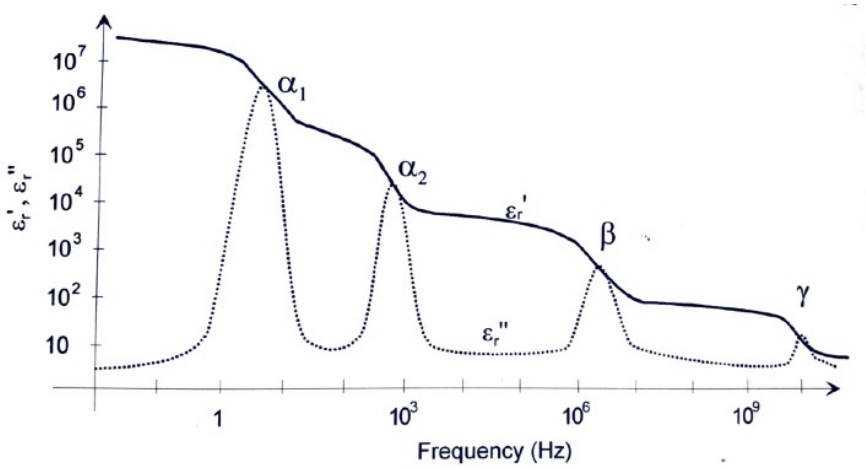


Figure 5. Dispersion regions for biologic tissue proposed by Schwan (Schwan, 1957).

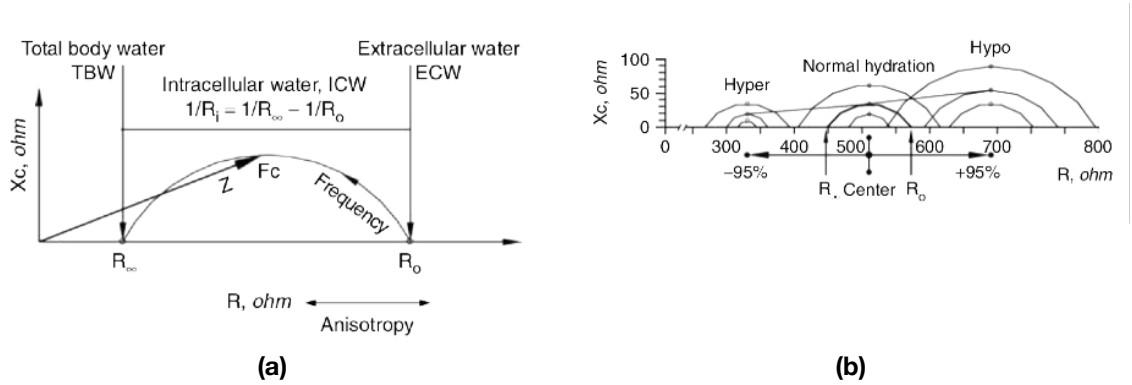


Figure 6. (a) In body composition analysis, the semi-circle of Cole's model for cell suspension is utilized. (b) The curve formed by Z vectors on the R-Xc plane (impedance locus) is a semicircle with a depressed center; smaller versus larger circles correspond to a more versus less hydrated status as progressive tissue dehydration increases tissue impedance. (R: resistance; R_0: resistance at 0 frequency; R_∞: at infinity; Xc: reactance). Retrieved from Piccoli et al. (2005).

1.2 Bioimpedance measurements

BIA measurements, depending on the position of the electrodes, can be useful to analyze the body composition of the whole-body or of some body regions. Measurements are made at 50 kHz, with the phase-sensitive analyzer BIA-101 Anniversary Sport Edition (AKERN-Srl, Florence, Italy) injecting a sinusoidal alternating current at 245 μARMS to measure R and Xc, with an error of system measurement, determined with a precision resistor and a capacitor (<1 Ω for R and <2% for Xc). The contact electrodes used (two for injecting of current I and two for detecting voltage V) are single-use pre-gelled Ag/AgCl Covidien Ref. 31050522 (Covidien llc, Mansfield, IL, USA) because they present very low intrinsic impedance (Nescolarde et al., 2016).

1.2.1 Whole-body bioimpedance

Four electrodes are placed (tetra-polar measurement) with the individual in supine position: two electrodes (an injector I, and a detector V) are placed in the dorsal face of 3rd right metacarpal's head and in the dorsal face of the right carpometacarpal joint, respectively; two more electrodes, an injector and a detector, are placed in the dorsal face of 3rd right metatarsal's head and in the dorsal face of the right tarsometatarsal joint, respectively (Figure 7).

With the configuration shown in Figure 7, under uniform hydration conditions (Foster and Lukaski, 1996; Houtkooper et al., 1996; Lukaski, 1996; Ellis, 2000; Grimnes and Martinsen, 2000), the total bioimpedance is composed of:

- 50% of total bioimpedance is determined by lower limbs.
- 40% of total bioimpedance is determined by upper limbs.
- 10% of total bioimpedance is determined by the rest of the body.

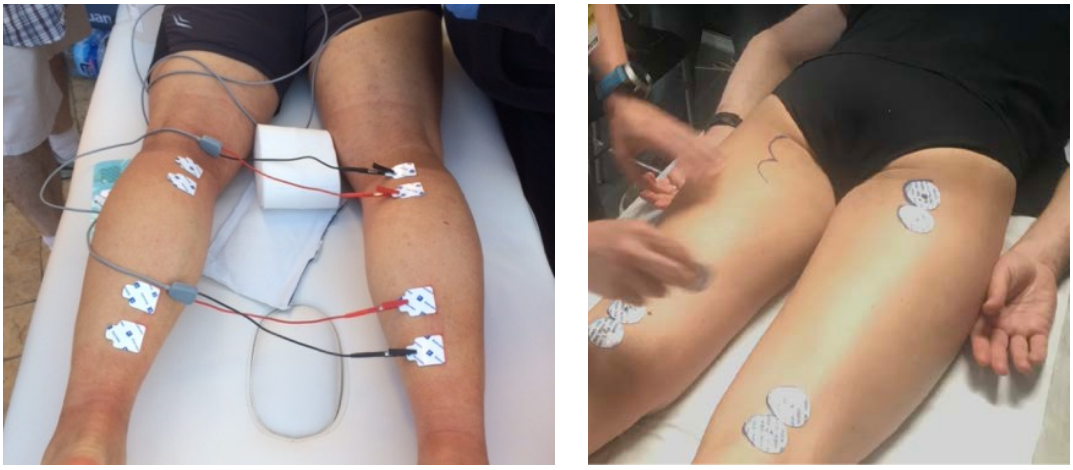
Figure 7. Electrode configuration, injectors and detectors (I,V), for standard measurement or whole-body.

Whole-body bioimpedance has the main objective to analyze hydration and nutrition changes in a non-invasive, fast, and repetitive method. For this reason there are many medical applications derived in different areas (Lukaski and Piccoli, 2012; Lukaski, 2013) like nephrology (Maioli et al., 2018; Nescolarde et al., 2004; Piccoli et al., 1994, 1995 and 1998; Tabinor et al., 2018), cardiology (Gastelurrutia et al., 2010; Massari et al., 2019; Nuñez et al., 2016; Santarelli et al., 2017), children and newborns (De Palo et al., 2000; Piccoli et al., 2002c), obese adults (Piccoli et al., 1998), patients undergoing peritoneal dialysis and hemodialysis (Nescolarde et al., 2008; Piccoli, 2004; Piccoli et al., 2005) among others. Also, in an elite sport where the nutritional condition and hydration is very important, whole-body BIA has been assessed. One example is in professional soccer players, showing how an increment in the resistance (R) is translated into dehydration and a decrease from the reference value is translated into a hydration status (Bonuccelli et al., 2011; Gatterer et al., 2011).

1.2.2 Localized bioimpedance

Another way to measure bioimpedance is by means of a localized four-electrodes measurement, known as L-BIA, where the two injector electrodes (I) and the two detectors (V) are placed on the area to be analyzed (Figure 8). This non-invasive method in the muscle assessment is supported by magnetic resonance imaging (MRI) and ultrasound (US) (Nescolarde et al., 2013, 2015 and 2017).

The L-BIA is useful because it measures the electrical variables of the changes that are produced by reactance (Xc), which identifies tissue destruction, and resistance (R), which identifies liquid content reorganization (Lukaski and Piccoli, 2012). For this reason it has been used as a non-invasive evaluation of muscle structure in patients with muscle atrophy (Rutkove, 2009), to identify regional alterations in fluid balance and soft tissue injuries associated with wounds in the lower part of the leg (Lukaski and Moore, 2012), and on the four main groups of the lower extremities to establish a pattern of muscle injury. In professional football players, L-BIA is used to assess muscle injury (Nescolarde et al., 2013 and 2015), and a follow-up until the return to play according to muscle gap (Nescolarde et al., 2017) finding a pattern of reduction in R and Xc directly related to the severity of the muscle injury according to cell disruption, being more pronounced this decrease (-12 to -56%) in the reactance (Xc). These findings highlight the sensitivity of L-BIA to identify fluid accumulation and muscle cell disruption.



(a) **(b)**

Figure 8. Location of the L-BIA electrodes in (a) twin muscle in Menorca Ultra Trail and (b) hamstring muscle in the Barcelona Marathon.

1.3 Bioimpedance measurement interpretation

Regarding body composition analysis based on impedance, two main interpretations can be found. Whole-body or right-side bioimpedance is the standard assessment, performed in the right side of the body, considering it as a cylinder with a given height and constant section area (Kushner, 1992; Lukaski et al., 1986). The conventional whole-body BIA equations are only accurate in healthy people, equations that are useful to estimate the body compartments like FFM, TBW, FM, ECW, ICW, among others (Nescolarde et al., 2008). Also, Bioelectrical Impedance Vector Analysis BIVA used in the classification and ranking of hydration status in conditions with perturbations in fluid balance is very expanded (Lukaski and Piccoli, 2012). One of the BIVA main advantages is its independence from the use of body weight and from any assumption about constant soft tissue hydration and chemical composition of the fat-free body mass. BIVA only depends on its reproducibility and accuracy measurements and the body composition intraindividual variability (Nescolarde et al., 2016).

1.3.1 Conventional bioelectrical impedance methods

The standard Bioelectrical Impedance Analysis (BIA) is measured between the right arm and the right leg as shown in Figure 7. Based on this measurement, body compartments (FFM, FM, ECW, ICW, etc) are estimated using multiple regression equations that include one of several terms related to the measured impedance and additional anthropometric terms like stature, weight, age, and gender.

There are equations to estimate volumes (intracellular, extracellular), mass (fat, fat-free, cellular), basal metabolism, and other variables (cellular Na/K, body density, etc). For example, total body water (TBW) is estimated using the impedance component R, the FFM is estimated assuming constant soft tissue hydration or the fat mass is calculated as the difference between the FFM and the body weight. In most of the regression equations, the Xc component is ignored (Ellis, 2000; Houtkooper et al., 1996; Piccoli et al., 2002b; Sun et al., 2003).

1.3.2 Bioelectrical Impedance Vector Analysis (BIVA)

The BIVA method (Piccoli et al., 1994) uses the measurement of the standard tetra-polar whole-body configuration as shown in Figure 7 (Lukaski et al., 1986). In contrast to other bioimpedance methods, BIVA does not yield any absolute estimate of ECW, ICW or TBW, makes no assumptions about body tissue isotropy or body models, hydration state, or the electrical model of cell membranes and it is unaffected by regression adjustments.

The impedance vector, the resistance R and the reactance Xc, are normalized by the height of the individual (R/H and Xc/H) and represented in the RXc graph (abscise R/H, ordinate Xc/H) (Figure 9). This technique allows the variations to be identified graphically with respect to the hydration of an individual or the amount of soft tissue, comparing the impedance vector with the distribution of the vectors of the reference population. The individual's vector is compared against the normal interval of the reference population, expressed in percentiles of 50%, 75% and 95% of the normal distribution (Gaussian) bivariate, probabilistic graph (Piccoli et al., 1994 and 1995). The correlation between R and Xc determines the ellipsoidal form of bivariate probability distributions (Lukaski and Piccoli, 2012).

If it is considered that the R is inversely proportional to the water content and the Xc directly proportional to the content of body cell mass contained in the soft tissues, the clinical interpretation of Figure 9 is as follows: variations in hydration, without an alteration of tissue mass-structure, are associated with a shortening (hyper-hydrated) or an enlargement (dehydrated) of the vector in the greater axis direction of the tolerance ellipses. On the other hand, variations in the structure of soft tissue are associated with a migration of the vector in the smaller axis direction of the ellipses, with either an increase in the phase angle (obese, athletic) or a decrease in it (lean, cachectic). Combined variations of hydration and nutrition are associated with the migration of the vector in the direction of the union of the two principal directions (Piccoli et al., 2002a).

The RXc graph method allows different evaluations of an individual vector:

- The parallel displacements to the major axis of tolerance ellipses indicate progressive changes in tissue hydration whereas displacements parallel to the minor axis are associated with changes in cell mass, contained in soft tissues.

Different trajectories indicate combined changes in both hydration and tissue mass.

- Evaluation of bioimpedance follow-up through the trajectory of the successive measurements of the impedance vector.
- Evaluation of groups of subjects using the bivariate 95% confidence ellipses of the mean vectors.

The applications of BIA measurements phase-sensitive at 50 kHz in the BIVA model are increasing, since apart from being used in the evaluation of global hydration, progress is being made in a variety of clinical conditions and in the evaluation of malnutrition, especially in diseases with muscle loss when hydration is altered. The position of the BIVA vector is easily interpreted as a normal or abnormal hydration based on the distance of the mean vector, but also its shortening or lengthening serves to interpret the response to the progression of a physiological process, a pathology or an intervention because it is reflected as a change in hydration (gain or loss of fluids) (Lukaski et al., 2019).

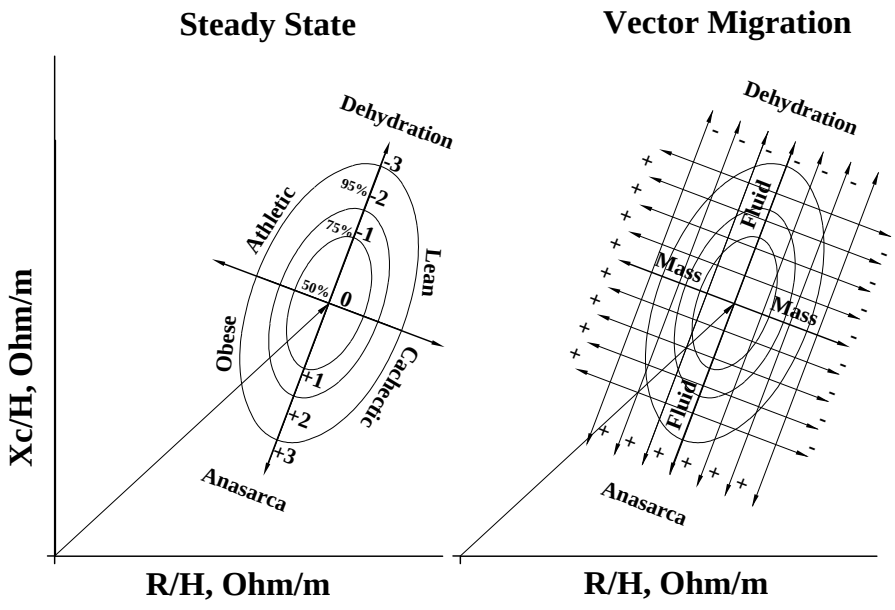


Figure 9. BIVA pattern (RXc graph). The three tolerance ellipses represent the 50th, 75th and 95th percentiles of the impedance vector bivariate normal distribution of a reference population of healthy caucasian males. Retrieved from Piccoli et al. (1994, 2002c).

2. Endurance exercise and skeletal muscle

2.1 Muscle tissue

Muscle is one of the four primary tissue types in the body and is responsible for most types of body movement, maintain postures, stabilize joints, and generates heat (Table 1).

Table 1. Main characteristics of Skeletal and Cardiac muscle (smooth muscle not shown). Adapted from Marieb, Human Anatomy and Physiology (2004).

	Skeletal muscle	**Cardiac muscle**
Description	Long, cylindrical, striated with multinucleate cells.	Branching, striated with generally uninucleate cells.
Function	Voluntary movement; locomotion; manipulation of the environment; facial expression; voluntary control	As it contracts, it propels blood into the circulation; involuntary control
Location	In skeletal muscles attached to bones or occasionally to skin	The walls of the heart

Skeletal muscle is the largest tissue in the body and is composed of a number of elongated cells known as fibers. The muscle fiber or myocyte grouped into fascicles is composed of parallel myofibrils (Figure 10), a sarcomere as the functional or contractile unit, a cell membrane called the sarcolemma, mitochondria arranged under this membrane and between myofibrils. The muscle cytoplasm is called sarcoplasm. In addition, the muscle fiber has multiple nuclei permitting the production of large amounts of proteins and enzymes needed for muscle contraction, and has a specialized smooth endoplasmic reticulum called sarcoplasmic reticulum that stores, releases, and retrieves calcium ions (Ca^{+2}) (Marieb, 2004). There are two main components of skeletal muscle that make the contractile function possible and efficient: myofibers and connective tissue. Each myofiber is attached at both ends to the connective tissue of a tendon or tendon-like fascia. The connective tissue provides the framework that binds the individual muscle cells during muscle contraction and embraces the capillaries and nerves within the muscular structure (Jarvinen, 2005).

The skeletal muscle is heterogeneous in nature and the selective recruitment of motor units enables a muscle to respond in the best manner to the functional demands. This heterogeneity is the base of the flexibility which allows the same muscle to be used for different tasks, for example, continuous low-intensity activity (posture), repeated sub-maximal contractions (locomotion), or fast and strong maximal contractions (jumping, kicking). In addition, due to the structural and functional properties of the fibers, the muscle has plasticity or malleability and can change in response to hormonal and neural influences, nerve-activity being a major determinant of the fiber type profile (see 2.1.1) (Schiaffino and Reggiani, 2011).

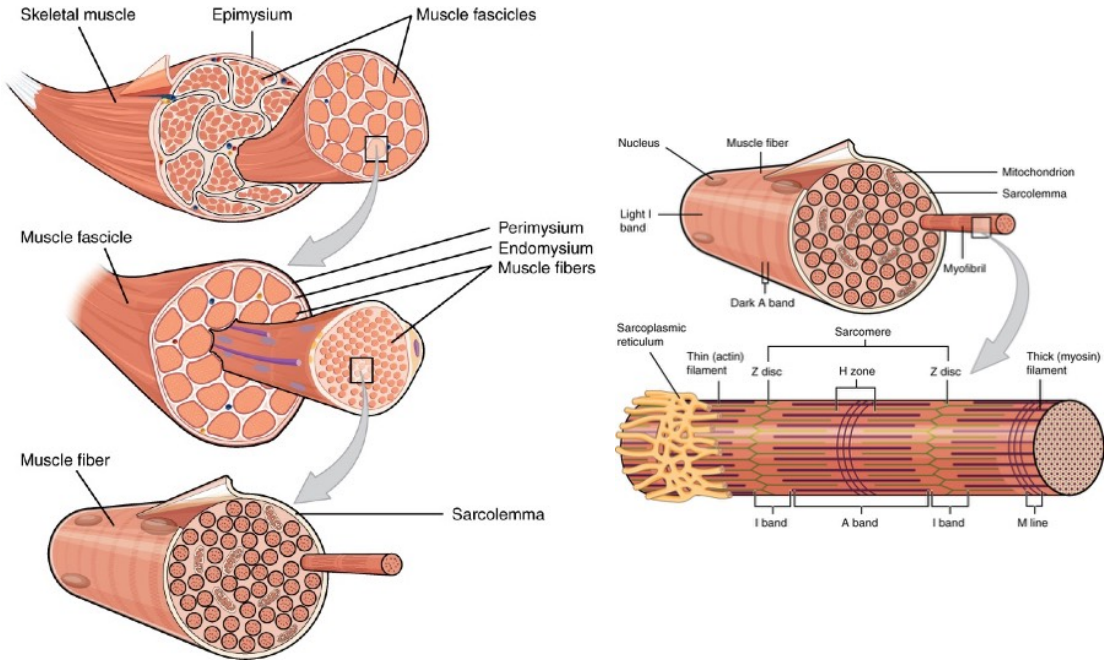


Figure 10. Muscle fiber organization. Retrieved from Hedegard and OpenStax, Anatomy and Physiology: Support and Movement (2014).

The striated muscles (skeletal and cardiac) begin the contracting process (shortening) when the actin protein (thin filament) is pulled by the protein myosin (thick filament) by cross-bridges between them. The contraction occurs after specific binding sites on the actin have been exposed in response to the interaction between calcium ions (released from the sarcoplasmic reticulum) and proteins (troponin and tropomyosin) (Figure 11).

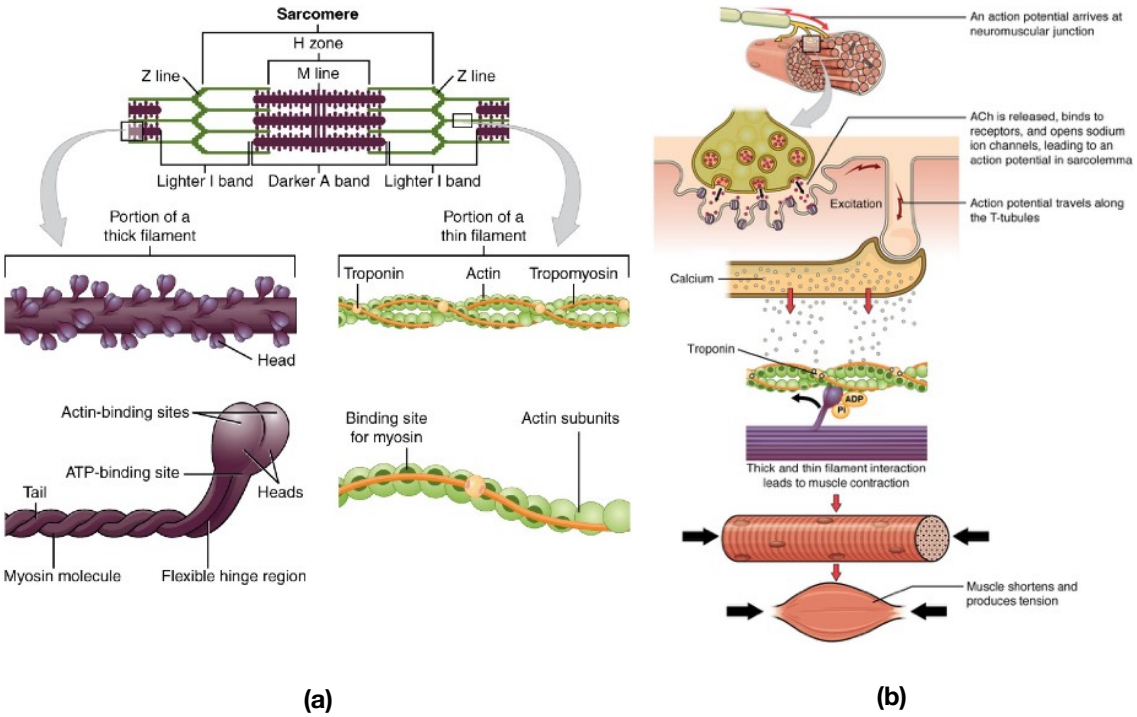


Figure 11. (a) Sarcomere structure with actin and myosin units as main proteins (b) Contraction sequence. Retrieved from Hedegard and OpenStax, Anatomy and Physiology: Support and Movement (2014).

The contraction cannot take place if ATP is not supplied as an energy currency. Thanks to ATP energy, the active-transport Ca^{+2} pumps in the sarcoplasmic reticulum can work. But the amount of ATP stored in muscle is very low (only sufficient to power a few seconds worth of contractions), and when it's used, it must be quickly regenerated and replaced to maintain a sustained contraction. Then there are three ways to regenerate ATP: creatine phosphate metabolism, anaerobic glycolysis, and aerobic respiration (Figure 12).

Thanks to the energy contained in phosphate bonds that the creatine phosphate molecule has, more ATP can be made for about 15 seconds. Creatine kinase (CK) is the enzyme responsible to catalyze the reaction that creatine phosphate transfers its phosphate back to ADP to form ATP and creatine (Figure 12a). But after this short period of time the muscle needs more ATP to maintain the contraction and then switch to glycolysis, an anaerobic (non-oxygen-dependent) process that breaks down glycogen to produce ATP (but not as fast as creatine phosphate does). The glucose used in this mechanism mainly come from glycogen stored in the muscle.

The two pyruvic acid molecules obtained with two ATP, can be used in the aerobic process converted to lactic acid (which quickly dissociates to lactate and hydrogen ion) (Figure 12b). Depending on the process then, glycogen from muscle reserves can go anaerobically to lactate or aerobically to pyruvic acid.

Aerobic respiration takes place in mitochondria with oxygen and is the breakdown of glucose and fatty acids to produce carbon dioxide, water, and ATP. This mechanism provides most of the energy needed for resting or moderately active muscles (Figure 12c). Another source of energy in long distance running are amino acids, which can be metabolized to produce glucose (Alberts et al., 2002).

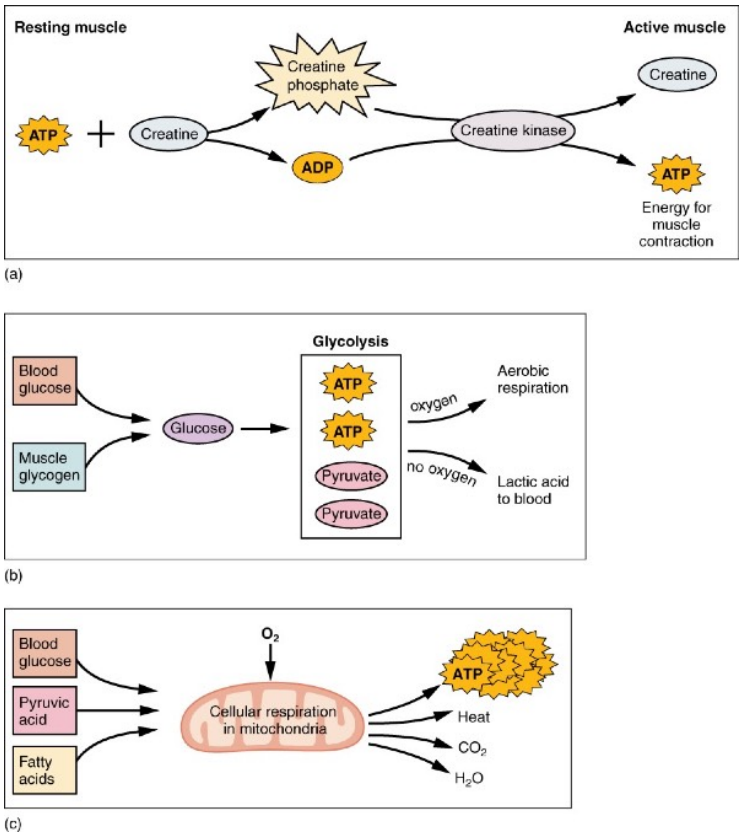


Figure 12. Three mechanisms by which ATP can be regenerated: (a) creatine phosphate metabolism, (b) anaerobic glycolysis and (c) aerobic respiration. Retrieved from Hedegard and OpenStax, Anatomy and Physiology: Support and Movement (2014).

2.1.1 Muscle training

There are large inter-individual responses to exercise training and huge differences in performance between individuals along with the role of genetic and epigenetic factors that affect them (Bouchard et al., 2011). During muscle contraction, there are tones of conversions of molecular events to chemical, electrical, and mechanical signals that promote physiological responses. These responses produce adaptations that can go from the activation and/or repression of specific signaling pathways regulating exercise-induced gene expression and protein synthesis and/or degradation (Hawley et al., 2014), to changes in contractile function, mitochondrial function, or metabolic regulation (Egan and Zierath, 2013). When someone is trained, moderate aerobic exercise produces only minimal increases in oxidative stress and inflammation, as opposed to the effects produced due to intensive training or excess exercise when one is poorly trained. This is partly because the training increases the activity of antioxidant enzymes and exerts a protective effect on cytoskeletal proteins (Brancaccio et al., 2010).

There are two basic types of training on which its effect is studied: endurance versus resistance exercise training. Endurance training is what we refer to as aerobic exercise (run, bike, walk, swim,...). In contrast, resistance training involves "pumping iron" and improving muscle strength and endurance. Resistance and endurance exercises induce different protein synthesis and depend on the training level. Resistance exercise training results in increased strength and muscle fiber cross-sectional area. Endurance exercise training is characterized by fatigue resistance due to increased oxidative capacity and mitochondrial density (Wilkinson et al., 2008).

In addition, it has been studied that the skeletal muscle has certain plasticity and adapts its response to a given exercise by converting one fiber into another. Different fiber types have been identified due to their contractile characteristics (they contain different myosin isoforms): type I (‘‘slow-twitch fibers’’ with high oxidative potential), type IIa (‘fast-twitch’ myofibers with high oxidative potential but a rapid fatigue profile) and type IId/x (primarily glycolytic) (Figure 13). Endurance training can change the myofibrillar content in muscle fibers by expanding mitochondrial and vascular networks (Rowe et al., 2014) or by enhancing the oxidative potential of type IId/x fibers (and thus significantly surpassing the aerobic capacity of type I fibers of

untrained persons) (Hawley et al., 2014). The pathways affected in fast or slow muscle fibers differ, specifically the metabolic fuel of glucose, lactate, and fatty acids (Figure 14). The variety of responses to exercise is a fact, each person has to act in consequence and program the right training for the objective to achieve, taking into consideration which fiber will be needed most.

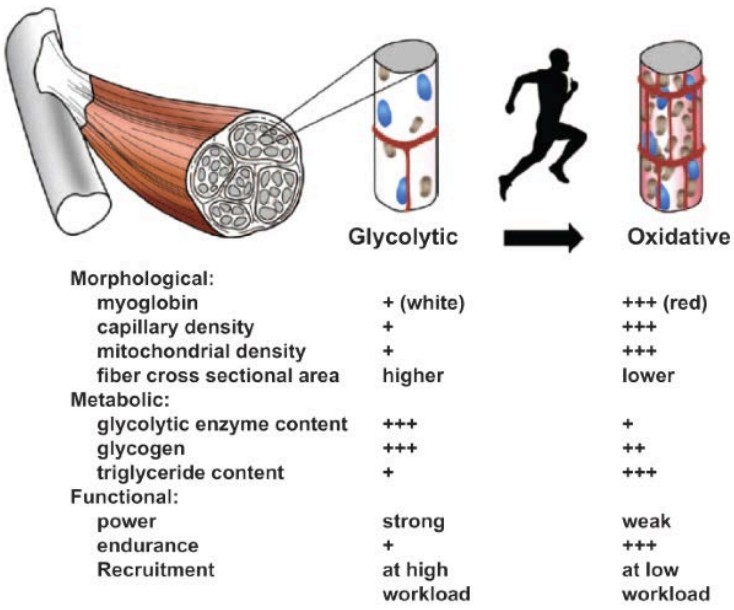


Figure 13. Outline of the muscle fiber types ranging from fast and glycolytic fibers to slow and mostly oxidative fibers. Retrieved from Rowe et al. (2014).

In addition to muscle typology there are other factors that determine the aerobic performance (Borresen and Lambert, 2009; Coyle, 1995):

- The economy of movement: defined as the oxygen cost to exercise at a given exercise intensity (velocity or $\%VO_{2max}$).
- The lactate threshold: defined as the exercise intensity (velocity or $\%VO_{2max}$) at which blood lactate increases significantly above the baseline level. Lactate threshold has been proposed as a measure of endurance fitness and also as a means to standardize training intensity, but it has limitations because its concentration depends on many variables. Lactate concentration can be influenced by exercise duration, intensity, rate of change, prior exercise, diet, muscle glycogen

content as well as exercising with damaged muscles, training status, and overtraining. In any case, the intensity of exercise required to induce optimal physiological adaptations for resistance events has been suggested as that which causes a lactate concentration of approximately 4 mmol/L. World-class athletes, for example, have a lactate threshold of 70-90% compared to 50-60% in untrained individuals (Finsterer, 2012).

- The individual perception of the effort conditions, the intensity and duration of the exercise, and is inherent to the physiological stress experienced during the exercise. It has been demonstrated how the Borg scale (RPE, rating of perceived exertion) is a valid measure of this perception (Foster et al., 2001).

A runner's performance is limited when they reach their maximum oxygen consumption (VO_{2max}), from which they can no longer burn more fuel for energy. VO_{2max} is defined as the maximum amount of oxygen that an athlete can absorb, transport and use to produce the energy needed to allow maximum intensity effort. For a healthy adult human at rest, whole-body oxygen consumption (VO_2) averages about 3.5 mL/kg/min which corresponds to one MET (metabolic equivalent of a task or to perform the activity). The 20%–25% of this consumption is used by resting the skeletal muscle, and for example a 70 kg person, whose resting O_2 consumption is 250 mL/min, the skeletal muscle will use 50 mL/min. In healthy untrained adults, VO_{2max} is typically 10–15 times resting values (35-53 mL/Kg/min), while in elite endurance-trained athletes, VO_{2max} values can exceed 85 mL/kg/min (Hawley et al., 2014). Some examples of incredible VO_{2max} in elite athletes can be the runner Kilian Jornet with 92 mL/kg/min, the cyclist Miguel Indurain with 88 mL/kg/min or the cross-country skier Bjørn Erlend Dæhlie with 96 mL/kg/min (TopendSports, 2019).

A runner with a VO_{2max} of 53 mL/kg/min, a maximal intensity in MET’s can be calculated using the equation:

Maximal Intensity = [53 mL/kg/min] / [3.5 mL/kg/min] = 53 / 3.5 = 15.14 METs

A MET is equivalent to 0.0175 Kcal/kg/min and thus it is possible to convert the METs to Kcal/min with the formula: Kcal/min = MET x 0.0175 x weight (kg).

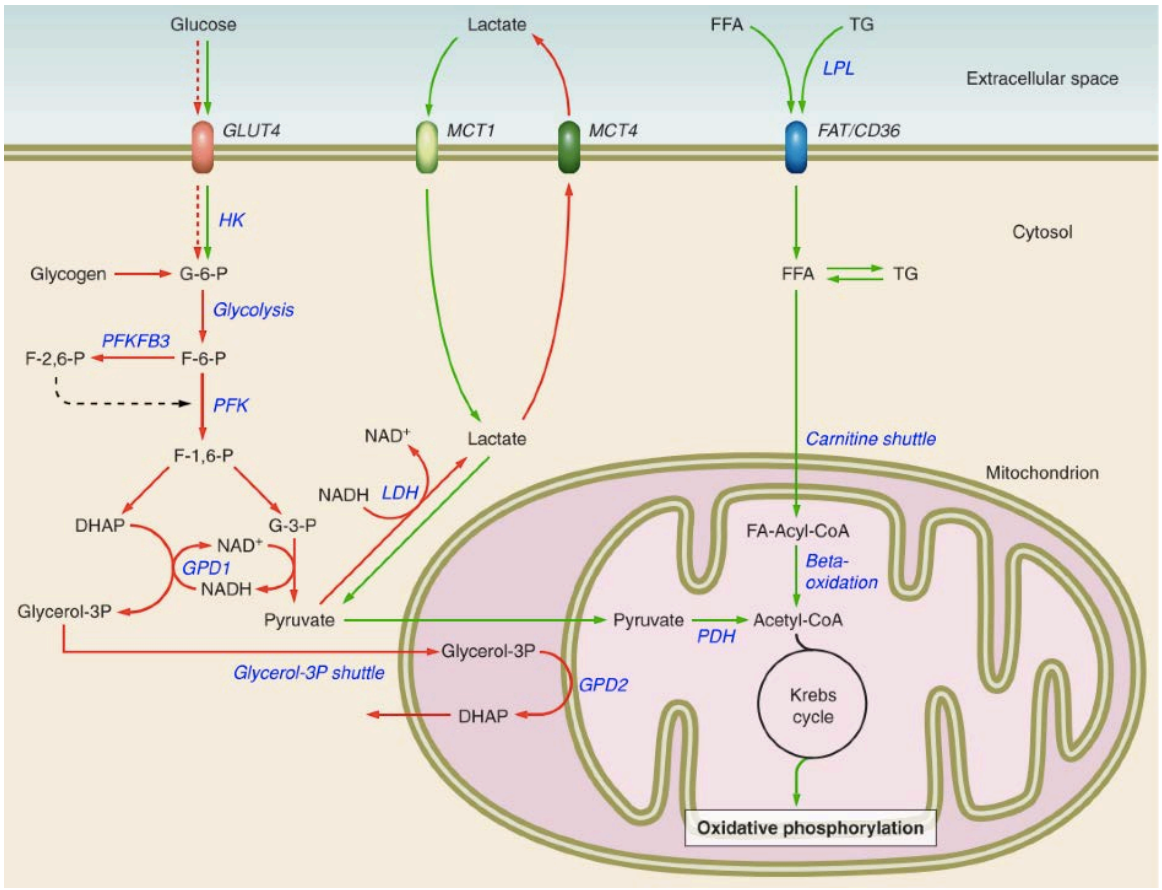


Figure 14. Pathways related to glucose, lactate, and fatty acid metabolism in fast (red arrows) or slow (green arrows) muscle fibers. DHAP, dihydroxyacetone phosphate; GLUT4, glucose transporter 4; F-6-P, fructose-6-phosphate; FAT/CD36, fatty acid translocase; FFA, free fatty acids; F-1,6-P, fructose-1,6-bisphosphate; F-2,6-P, fructose-2,6-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; G-6-P, glucose-6-phosphate; GPD1, glycerol-phosphate dehydrogenase 1 (cytoplasmic); GPD2, glycerolphosphate dehydrogenase 2 (mitochondrial); HK, hexokinase; LDH, lactate dehydrogenase; MCT1, monocarboxylic acid transporter 1; MCT4, monocarboxylic acid transporter 4; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PFKFB3, phosphofructokinase-fructose bisphosphatase 3; TG, triglycerides. Retrieved from Schiaffino and Reggiani (2011).

Each physical activity and depending on the intensity has assigned specific METs (Table 2), for example 1 MET is the rate of energy expenditure while at rest and 4 MET activity expends 4 times the energy used by the body at rest. MET values of activities range from 0.9 (sleeping) to 23 (running at 22.5 km/h or a 4:17 mile pace) (Ainsworth et al., 1993 and 2000).

Table 2. MET metabolic equivalent task of some main activities and its equivalent in Kcal/min for a person with 70 kg weight. mph, miles per hour. Adapted from Ainsworth et al. (1993, 2000).

Type of activity	Activity characteristics	MET	Kcal/min (person of 70 kg)
Inactivity	sleeping	**0,9**	**1,10**
	lying quietly	**1**	**1,23**
Home activities	cleaning house or cabin	**3**	**3,68**
Walking	backpacking	**7**	**8,58**
	downstairs	**3**	**3,68**
	hiking, cross country	**6**	**7,35**
	3 mph, moderate pace, firm surface	**3,3**	**4,04**
Bicycling	<10mph, general,leisure	**4**	**4,90**
	14-15,9 mph, racing or leisure, fast, vigorous effort	**8**	**9,80**
	>20 mph, racing, not drafting	**16**	**19,60**
Running	jogging	**7**	**8,58**
	6 mph	**10**	**12,25**
	10 mph	**16**	**19,60**
	cross-country	**9**	**11,03**
	stairs, up	**15**	**18,38**

2.1.2 Muscle fatigue

Physical fatigue is the transient inability of a muscle to maintain optimal physical performance. During physical exercise, fatigue is a multifactorial symptom that may be related to both central and peripheral factors, all of which can be influenced by the intensity and duration of the exercise, the nutritional intake, and the training status of the individual (Blomstrand, 2006).

Peripheral fatigue can be due to depletion of muscle glycogen (chronic fatigue) or phosphocreatine, accumulation of protons from acid lactic (acute fatigue), and failure of neuromuscular transmission. Muscle function is reduced when ATP reserves (needed for normal muscle contraction) decline. The induced acidosis of lactic acid can accelerate fatigue during high-intensity exercise in events that last from 1 to 10 minutes, but in marathons and ultramarathons lactic acid provides benefits. This lactic acid or lactate/H^+ can be converted back into pyruvic acid to be metabolized with oxygen, or it can be reconverted into glucose mainly at the renal and hepatic level, and also this glucose can replenish glycogen stores in the muscles (Cairns, 2006).

Central fatigue due to neurobiological factors is less well known. During the prolonged exercise of moderate intensity, fatigue comes when blood glucose levels decrease. This decrease is due to a depletion of the liver glycogen stores or an increase in neurotransmitter release like 5-hydroxy-tryptamine (5-HT). It has been studied how a BCAA ingestion can delay fatigue due to a tryptophan decreased transport into the brain with a decrease in 5-HT synthesis (Blomstrand, 2006). Also, there is a ‘‘central governor model’’ (CGM) that can explain fatigue under all of the exercise conditions that have previously been attributed to task dependency as a general model of fatigue. This CGM explains how the subconscious brain determines the number of motor units that are activated and thus determines the intensity of the exercise. When there is a difficulty in maintaining homeostasis at a given intensity of exercise, the subconscious brain informs the conscious brain that it interprets it as an increase in the sensation of fatigue (Weir et al., 2006). When a runner’s oxygenation reaches the limits of what is safe, the brain’s motor cortex (which recruits the exercising muscles) stops recruiting additional muscle and the body experiences fatigue. The work output stopped by muscle and heart, reduces the

heart oxygen demand and protects the body from damage caused by oxygen starvation (Noakes, 2002).

2.1.3 Muscle damage and injury

If we analyze only the adverse effects when we practice moderate aerobic exercise, there are two main types of risk: the risk of injuries in the locomotor system (0.19 to 1.3 injuries per 1000 hours of physical exercise is observed, which is equivalent to one injury per person every 4 years) and the cardiovascular risk (sudden death and acute myocardial infarction, generally in people with underlying structural heart disease) (Subirats et al., 2012). But marathon and ultramarathon events are not moderate aerobic exercises, they are severe bouts of running exercise that comprises a distance of 42.195 km or more. Unlike marathons, ultramarathons occur in more extreme environments with variations in terrain, temperature and humidity, particular factors that may contribute to injury and illness (Krabak et al., 2013). More than 25% of the new runners face an ultramarathon in a period less than three years after starting, which leads to possible health problems that can become serious. For example, there are more stress fractures involving the foot especially in less experienced runners (Hoffman and Krishnan, 2014) and there are lower extremity running injuries predominantly in the knee that can be reduced by an increase in training distance per week (Gent et al., 2007). Regarding an asphalt marathon, there is eccentric hamstring fatigue that may be a potential risk factor for knee and soft tissue injuries during the run (Koller et al., 2006), these injuries are very common among recreational male marathoners (Middelkoop et al., 2008). It has been seen that training diminishes the muscle fiber injury adding sarcomeres to regenerate fibers (Proske and Morgan, 2001) or protecting the mitochondria integrity (Carmona et al., 2018). For this reason, training is a powerful tool to protect us.

According to Guerrero et al. (2008), our muscles are made up of a mixture of slow and fast-twitch fibers with approximately 50% of each, very different compared to animals, which have only one fiber predominantly, and fast fibers are more easily fatigued and more sensitive to injury. Even though the skeletal muscle has certain plasticity and adaptability, numerous issues relating to the speed, force, duration, and intensity of muscle contractions, along with the total muscle mass engaged in the activity, are important for a complete understanding of the physiological responses to exercise. A sarcomere disruption, a loss of sarcolemma integrity, a loss

of mitochondrial integrity, an increase of membrane permeability, a disintegration of the Z-discs, contracture knots, and/or accumulations of erythrocytes and phagocytes within the muscle fibers lead to the presence of enzymes in the blood that are normally located in muscle fibers.

The muscle damage that the runners can suffer, increase both with intensity and duration of the exercise, with intensity the greater influence (Armstrong, 1986). The symptoms of muscle pain (from 1 to 7 days following the race), spasms or some swelling, may occur as a result of injury or when the muscle produces more physical work than it is used to produce (Brancaccio et al., 2010). But before a muscle is injured, there are three main phases of muscle damage that can occur with exercise (Noakes, 2002):

1st: After hours of unaccustomed exercise in trained athletes there's a leakage of moderate amounts of muscle enzymes into the bloodstream, peaking at 24-48 h after exercise.

2nd: After 4 to 6 days of unaccustomed eccentric exercise in untrained athletes there are much higher rates of enzyme leakage into the blood, infiltration of muscle by inflammatory cells, and degeneration of muscle cells, with the latter peaking 10-12 days after exercise. Initiation of an inflammatory response 24-48 h after exercise, stimulates nerve endings in the damaged tissue, causing the typical pain of delayed soreness. The Z-band of the sarcomere is the most affected and its disruption provokes a release of protein breakdown products that lead to fluid accumulation and swelling of the muscles, then the intramuscular pressure elevates and increases the muscle soreness.

3rd: Tissues can be damaged by the free radicals released when the oxygen is used actively. These radicals attack cell membrane lipids and damage them in a peroxidation reaction.

The most common muscle injury to marathoners are (Noakes, 2002):

- Delayed onset muscle soreness (DOMS): muscle discomfort 24 to 48 h after unaccustomed or particularly severe exercise, especially if it involves eccentric muscle contractions, due to muscle damage, in particular, the connective tissue and the contractile proteins.

- Exertional muscle cramps tend to appear in people who run farther or faster than the distance or speed to which they are accustomed to. Only muscles that undergo lengthening frequently during prolonged exercise may be prevented from cramping. Runners with cramps don't have gross disturbance in blood electrolyte levels, and they are not more dehydrated than the runners without cramps.

As Armstrong (1986) and Noakes (2002) described, 3 proposed mechanisms work in concert to produce DOMS following eccentric exercise: structural damage to muscle, disruptions in calcium homeostasis, and/or sensitization of type IV nerve endings by means of the products of invading inflammatory cells. But recent reviews of muscle inflammation post-injury provide more comprehensive details of this complex and highly coordinated response (Figure 15) (Hyldahl and Hubal, 2014).

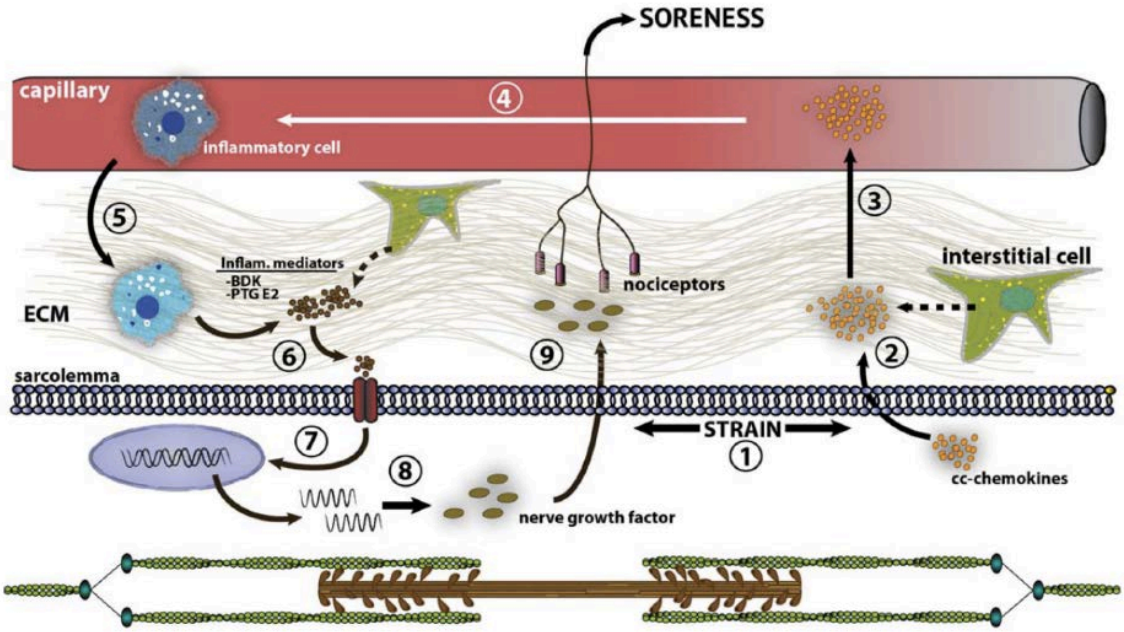


Figure 15. Theoretical framework summarizing possible mechanisms for delayed muscle soreness following eccentric exercise. In response to strain (1) from eccentric actions, cc-chemokines and other signaling proteins are produced and released (2) from damaged or stimulated myofibers. Also chemokine release may be from interstitial cells residing in the extracellular matrix (dotted line). Chemokines enter the circulation (3), and recruit inflammatory cells (4). Inflammatory cells infiltrate the skeletal muscle (5) and release chemical mediators such as bradykinins (BDK), and prostaglandins (PTG E2) (6), which may act directly on muscle nociceptors to produce muscle soreness, or bind to extracellular receptors (7) and up regulate the expression of proteins (i.e., nerve growth factor) (8) that are excreted from the muscle fiber and act on muscle nociceptors to produce soreness (9). Retrieved from Hyldahl and Hubal (2014).

2.2 Muscle blood biomarkers

One of the ways to objectively measure physiological or pathogenic processes in the body that occur during a marathon is through biomarkers (biological markers). These are measurable products or substances of an organism that are used as indicators of a biological state. Specifically in this chapter, the biomarkers assessed are from the cytosol (not from the cell structure) and indicate a change in the expression or state of a protein that correlates with muscle damage and inflammation.

2.2.1 Muscle damage biomarkers

When exercise intensity exceeds the aerobic range, membrane permeability changes and enzymes are released. When a load of muscle tissue exceeds a certain limit, creatine kinase (CK) and other intracellular proteins are filtered in the interstitial fluid, absorbed by the lymphatic system and returned to the circulation (Brancaccio et al., 2010).

Creatine kinase

Creatine kinase (CK) is a dimeric globular protein with a molecular mass of 43 kDa for each subunit. As seen in Figure 12a, it catalyzes the reversible exchange of high-energy phosphate bonds between phosphocreatine and ADP produced during contraction.

At least five isoforms of CK exist and are tissue-specific: three isoenzymes in the cytoplasm (CK-MM, CK-MB and CK-BB) and two isoenzymes (non-sarcomeric and sarcomeric) in mitochondria. On injured tissues, CK isoenzymes can give specific information because of their localization. In fact, CK-MM is found in several domains of the myofiber where ATP consumption is high and is a marker of muscle disease, CK-MB increases in acute myocardial infarction, CK-BB increases in brain damage and mitochondrial CK is raised in mitochondrial myopathies (Brancaccio et al., 2007).

Since the beginning, creatine kinase found almost exclusively in muscle tissue (skeletal and cardiac), has been considered the best indicator of muscle damage after an exercise such as a marathon (Apple et al., 1985). The reason is mainly mechanical because it starts with the damage of the structure of the skeletal muscle

cells at the level of sarcolemma and Z disks as already mentioned in section 2.1.3. Normally, only CK-MM is present in the serum, but prolonged and strenuous exercise increases the serum activity of all three CK-isoenzymes in the absence of myocardial damage. As total CK is not fiber-type-specific, other biomarkers have been used in order to characterize the exercise impact on muscle fibers, like slow and fast myosin as indirect biomarkers of fiber-specific sarcomere damage (Carmona et al., 2018).

CK activity is markedly elevated for 24 h after exercise, remains elevated for 48 h and can be pronounced between 2-7 days when there is muscular damage due to an eccentric exercise. This elevation after exercise is usually lower in trained individuals when compared with untrained ones, but also depends on age, gender, race, muscle mass, physical activity, and climatic condition. In addition, a large increase in serum CK levels combined with reduced exercise tolerance could be an overtraining marker (Brancaccio et al., 2007).

In the context of marathons and ultramarathons, some studies have reported that over half of marathon participants have elevated unspecific creatine kinase 24 h after, which can exceed 2400 IU/L (remaining 15-fold above baseline for over a week) and in ultramarathon distances, nearly 6-10% of the runners recorded values >100,000 IU/L (Bird et al., 2014) and without apparent health consequences (Hoffman et al., 2012).

With all the aforementioned, a certain degree of skeletal muscle breakdown is expected from endurance exercise, especially if this exercise is longer or more intense than a runner is accustomed to. But when many skeletal muscle cells break apart, they release CK along with electrolytes like potassium and calcium, and myoglobin, the protein from the muscle tissue. The precipitation of this protein in blood can damage the kidney blocking the tube-like filters and then electrolytes can build up in the blood causing cardiac arrhythmias. This “striated muscle breakdown” due to exercise is called exertional “rhabdomyolysis”, and even though it appears in marathon and ultramarathon runners, only a few cases are clinically significant (even with CK levels greater than 100,000 IU/L). Signs of clinically significant rhabdomyolysis are: generalized muscle pain or weakness out of proportion to the effort, dark-colored urine or the inability to urinate 12-24 h post-race. Risk factors for exertional rhabdomyolysis are: unaccustomed and/or eccentric exercise, heat/cold,

dehydration/over hydration, nonsteroidal anti-inflammatory drugs (NSAID) and other medication use, current infection, male sex and genetic predisposition (Scalco et al., 2016). Some ways to avoid rhabdomyolysis are specific training and proper hydration.

Lactate dehydrogenase

Lactate dehydrogenase (LDH) is a tetramer with a molecular mass of 144 kDa. It is a hydrogen transfer enzyme that is found in the cytoplasm of most body cells and its isoenzymes are tetramers of either heart (H) or muscle subunits (M). All tissues contain various amounts of the 5 LDH isoenzymes; however, muscle, liver, and red blood cells are the major sources of serum LDH activity (Markert, 1984).

LDH is a glycolytic enzyme that catalyzes the conversion of lactate to pyruvate, whereas NAD^+ is reduced to NADH or vice versa, during glycolysis in conditions of hypoxia. It is found in almost every tissue, especially in skeletal muscle, heart, liver, kidneys, brain, lungs, and red blood cells.

Serum LDH activity is a marker of cell/tissue damage, and the specific increase in isoenzymes may be helpful for the diagnosis in non-traumatic acute rhabdomyolysis. Its elevation in serum can contribute to muscle and liver damage, common injuries and disease such as heart failure and cardiac stress (Lippi et al., 2011). Also, exercise induces a significant increase in LDH and the degree of this increase depends on the intensity and duration of the effort and its peak can be observed within 48 to 72 h after the effort. After prolonged endurance exercise like a marathon, the LDH activity can be doubled and remain increased for 2 weeks (Brancaccio et al., 2010).

The normal range of serum LDH in a healthy adult is 50-150 U/L, and after a marathon especially when the pace is reduced due to muscle damage, LDH values can increase nearly to 700 U/L (Del Coso et al., 2013) or 1000 U/L after an ultramarathon (Shin et al., 2016).

2.2.2 Inflammation biomarkers

C-reactive protein

C-reactive protein (CRP) is a member of the pentraxin proteins with a molecular mass of 118 kDa. It has five non-covalently bonded and non-glycosylated identical subunits of 206 amino acids each that form a disk-shaped pentagon.

CRP is a pattern recognition protein found in blood plasma and it is an integral part of the innate immune system. It was discovered by Tillet and Francis in 1930 in patients with acute pneumococcal pneumonia, where CRP interacted with the C-polysaccharide of streptococcus pneumoniae cell wall (hence the term C-reactive protein) (Bray et al., 2016).

CRP is produced and synthesized in the liver in response to inflammatory cytokines and assists in complement binding and phagocytosis by macrophages. Its physiological role in innate immunity as an early defense system against infections is to bind to lysophosphatidylcholine expressed on the surface of dead or dying cells (and some types of bacteria) in order to activate the complement system (Thompson et al., 1999; Bray et al., 2016). CRP coordinates the host's immune response against pathogens and damaged tissues as a class of acute-phase reactant and the first pattern recognition receptor (Figure 16). Pathogen-associated molecular patterns (PAMPs) drive nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and other inflammatory pathways by surface-expressed toll-like receptors (TLR) and induce the release of inflammatory cytokines from macrophages (Figure 16 left). In a parallel activation pathway, major tissue injury activates the “sterile inflammation pathway” by the release of damage-associated molecular patterns (DAMPs) to activate the inflammasome-mediated processing of IL-1β and IL-18 after binding to endosomal receptors (Figure 16 right). Finally, CRP binds to target tissues via Fc receptors or other binding molecules such as M-ficolin (Vashist et al., 2016).

The normal concentrations of CRP in a healthy adult varies between 0.8 mg/L to 3.0 mg/L, but they can rise 10,000-fold after a stimulus with peaks at 48 h. The only factor that affects the level of serum CRP is its rate of production because the plasma half-life is 19 h. This rate of production increases with inflammation, infection, trauma, tissue injury, necrosis, malignancy, and allergic reaction (Pepys and Hirschfield, 2003).

CRP is one of the proteins involved in physical stress reactions, implicated in the cardiovascular system and renal function. Elevated levels of CRP are directly associated with cardiovascular risk, atherothrombotic disease, acute myocardial infarction, and acute coronary syndrome. Associations of CRP with cardiovascular disease, for example, are due to the selective binding of CRP with LDL, depositing in most atherosclerotic plaques with the activated complement (Pepys and Hirschfield, 2003). The muscle cell damage after a marathon results in the transient rise of serum creatinine that meets the clinical criteria of acute kidney injury (AKI) (Bekos et al., 2016). CRP is considered a biomarker of inflammation in AKI, impairing tubular regeneration and promoting fibrosis of injured renal tissue (Tang et al., 2014 and 2018). It remains unknown the impact of repetitive episodes of AKI on long-term kidney function.

After prolonged exercise, CRP serum concentrations start to rise within 8 h after exercise and peak 24 h after. This rise depends on the exercise duration; running events of up to 21 km cause only a marginal elevation in serum concentrations while a marathon or ultramarathon result in much higher levels. The CRP levels found after a 90 km ultramarathon are similar to those measured in persons with mild heart attacks (Noakes, 1987). Some studies showed how CRP plasma levels increased 3.4-fold, one day after a marathon and returning to the pre-race level on day 4, 20-fold after a 168 km ultramarathon (Waskiewicz et al., 2012), or 40-fold after a 200-km race and was still significantly higher even after day 6 of recovery (Kim et al., 2009).

Running a marathon or an ultramarathon leads to a substantial amount of skeletal muscle damage and an acute inflammatory response evidenced by a wide range of changes in muscle damage-related biomarkers and increased CRP levels as seen in this thesis.

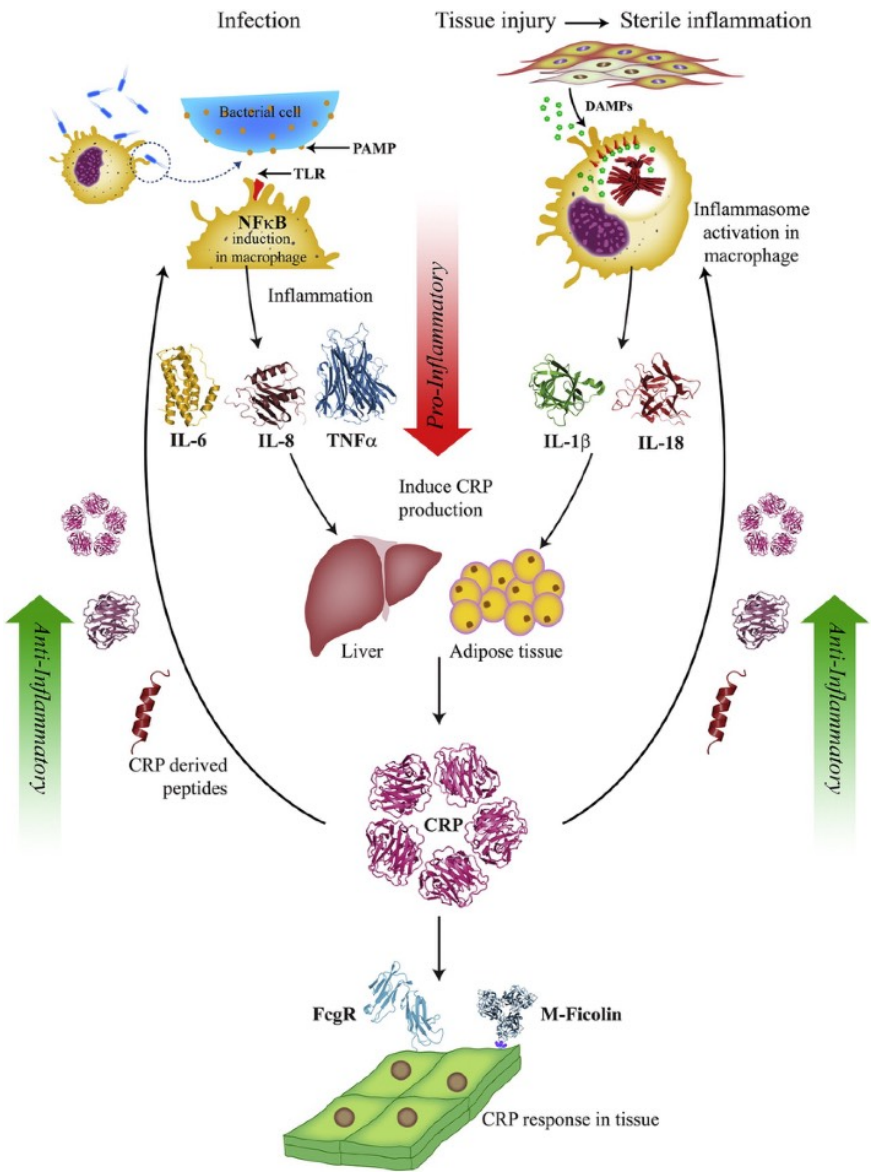


Figure 16. The major pathways related to the CRP production. Retrieved from Vashist et al. (2016).

2.3 L-BIA measurements

In general, the most common mechanism of injury of muscles in the lower limbs (Gent et al., 2007) is related to muscle strain with muscles being at risk of disruption during eccentric contraction. Eccentric exercise performed when a runner is dehydrated, for example, may exacerbate the skeletal muscle damage as a result of reduced intracellular water (Cleary et al., 2006). Different studies have found that repeated eccentric muscle contractions produce progressive muscle damage reducing its power output (Hyldahl and Hubal, 2014), and this form of fatigue is showed when runners hit the wall in marathon and ultramarathon races (Noakes, 2002). Usually, a marathon run is associated with eccentric hamstring fatigue (Koller et al., 2006), being the hamstring strains one of the most frequently occurring injuries in mid- and long-distance runners, affecting 12–16% of all injuries in athletes (Chu and Rho, 2016) (Figure 17). Acute hamstring strains can occur with high-speed running or with excessive lengthening of the hamstrings, especially in runners without enough flexibility, strength and core stability, or due to a muscle fatigue (Chu and Rho, 2016). The L-BIA measurements are a non-invasive alternative to evaluate runners with high and continuous muscle stress and prevent running muscle injuries like the hamstring ones.

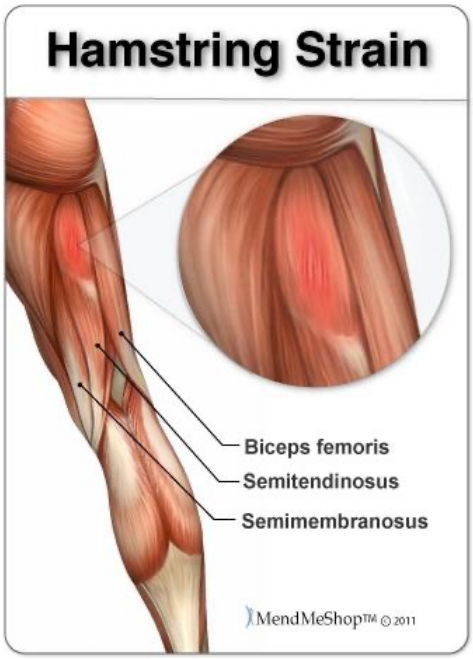


Figure 17. Drawing of muscle damage due to hamstring strain. Image retrieved from http://www.aidyourhamstring.com/hamstring-pain/hamstring-strain.php .

3. Endurance exercise and cardiovascular health

The heart is a muscle of only 250 grams that works tirelessly day and night, beating 100,000 times and driving 8,000 liters of blood through more than 160,000 km of blood vessels in our body every day. The heart has two very effective activities, one electrical with stimulation, and the other one with contraction, and its coordination must be perfect. The electrical activity, both of the atria and the ventricles, can be measured with the electrocardiogram, and mechanics with imaging techniques, such as echocardiography.

The heart of the athlete presents adaptive changes, when it contracts it expels more blood in each beat and has a lower heart rate, thus having a longer ventricular filling time. Cardiac output at rest is approximately 5 L/min but increases 5-fold to about 25 L/min during vigorous exercise. In response to chronic physical exercise, the heart experiences structural and functional changes that are especially related to the intensity and duration of this exercise and therefore a medical evaluation is essential to ensure that the heart can withstand it.

Numerous epidemiological studies suggest that regular physical activity (or moderate to high levels of cardio-respiratory fitness) protects against cardiovascular diseases (CVD), but the intensity of this exercise can trigger the risk, especially in those individuals with known or occult CVD (Franklin, 2014). Frequent high-intensity exercise could be associated with negative health impact and results from sub-analysis of the Copenhagen City Heart Study suggested that jogging at a slow to moderate pace not more frequent than three times a week was associated with the lowest mortality, while no survival benefit was seen when jogging at a fast pace, or more than three times a week (Schnohr et al., 2013).

At the end, it is a question of progression during the physical activity and common sense, and for sure a medical evaluation is urgent for those who plan to remain inactive, but also for those more than 35 years old, who want to be in shape (Figure 18).

Sudden cardiac death in apparently fit athletes mostly occurs in the absence of any warning symptoms or a history of heart disease (Finocchiaro et al., 2016). The current

American Heart Association (AHA) recommendations related to a pre-participation screening to identify the presence of silent heart diseases can reduce the risk of sports-related sudden death, like personal history, physical examination, and electrocardiogram (ECG) test (Maron et al., 2007). The addition of 12-lead ECG in the screening can increase the sensitivity in detecting significant silent cardiac abnormalities, but there is a divide in North America versus Europe guidelines regarding incorporating ECG as a mandatory test for pre-participation screening (Vora et al., 2018). According to Corrado et al. (2006 and 2011) ECG in pre-participation screening in young athletes reduced 90% of the sports-related mortality, however, in a study of Steinvil et al. (2011), ECG was not associated with a reduction in adverse cardiac events. In the SUMMIT project, for example, an ECG before an ultramarathon saved one runner's life, finding severe atrial fibrillation.

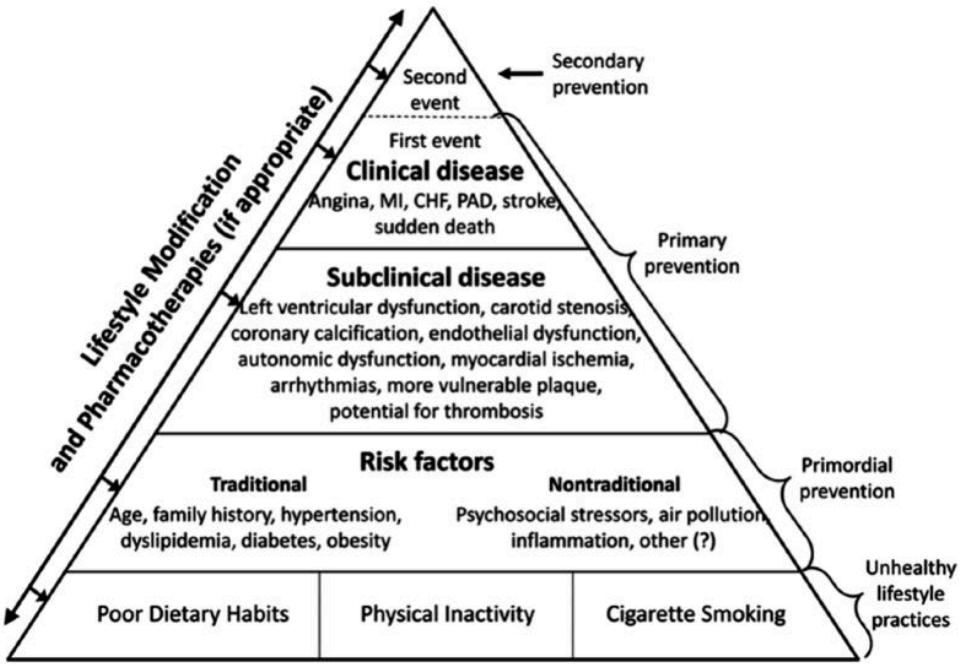


Figure 18. The evolutionary cardiovascular disease pyramid. The way to prevent initial or recurrent cardiac events is to modify unhealthy lifestyle habits that are the base of the pyramid (CHF: congestive heart failure; MI: myocardial infarction; PAD: peripheral arterial disease). Retrieved from Franklin (2014).

3.1 Cardiac muscle

Cardiac muscle tissue is only found in the heart and even though it is similar to skeletal muscle (striated and organized into sarcomeres), the cardiac tissue fibers are shorter, contain a nucleus and have active mitochondria and myoglobin because ATP is produced primarily through aerobic metabolism (Figure 12c). Cardiac muscle fiber cells are extensively branched and connected by intercalated discs, which are part of the sarcolemma and contain two structures important in cardiac muscle contraction: gap junctions and desmosomes. The heart can work as a pump because these discs allow the cardiac muscle cells to contract in a wave-like pattern (Figure 19). Heartbeats are controlled by specialized cardiac muscle cells called pacemaker cells that directly control heart rate. These cells cannot be consciously controlled, they respond to signals from the autonomic nervous system (ANS) to speed up or slow down the heart rate and can also respond to various hormones that modulate heart rate to control blood pressure.

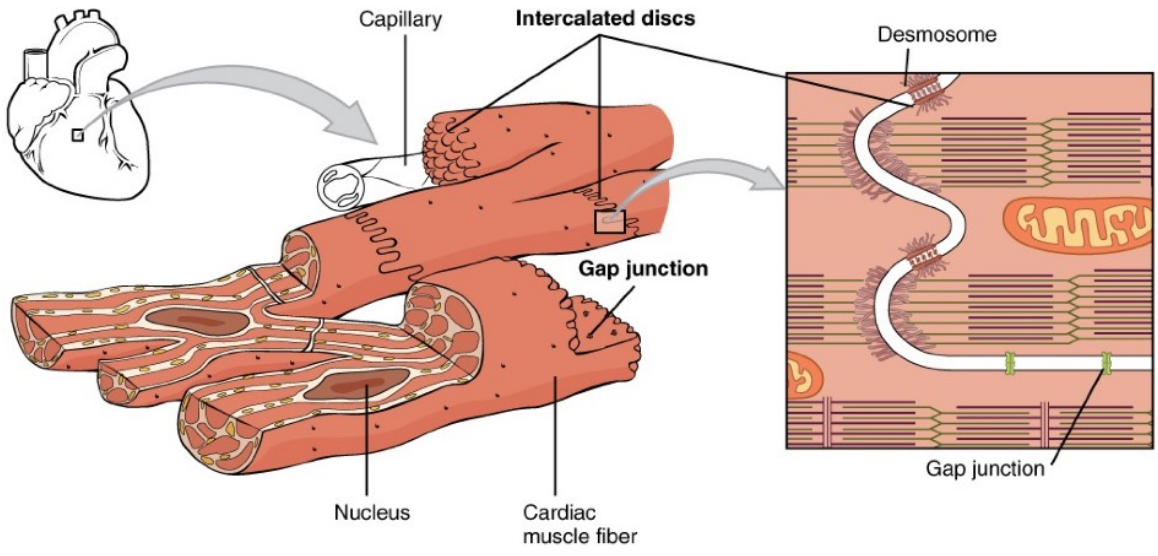


Figure 19. Cardiac muscle and its intercalated discs with gap junctions and desmosomes. Retrieved from Betts et al. and OpenStax, Anatomy and Physiology: Cardiac Muscle Tissue (2017).

3.2 Athlete’s heart

The effects of training are difficult to define clearly, but in general, it is accepted that bradycardia is at rest, lower systolic and diastolic blood pressure and the increase in the size of the cardiac cavities (Mitchell and Raven, 1994). The heart is probably the organ that supports greater overload during the practice of physical exercise, undergoing notable changes both morphological and functional, which is classically referred to as the athlete's heart. This cardiac remodeling is the process resulting from changes in the geometry and structure of the myocardium, and, in some cases, the appearance of fibrotic tissue accompanied by alterations in myocardial contractility.

There are 2 types of enlarged hearts. The first type, athlete’s heart, is caused by normal muscle growth in the heart due to the practice of vigorous sports and does not put the athlete at risk. However, the second type, hypertrophic cardiomyopathy, is a very risky disease and can cause malignant heart arrhythmias that can lead to sudden death (Figure 20).

Athlete’s heart with a normal cardiac function is the result of reversible physiological remodeling resulting in increased cardiac mass (Weeks and McMullen, 2011). There are slight differences in the extent of remodeling based on the type of exercise. In endurance runners, for example, there is eccentric myocardial hypertrophy due to volume overload, the left ventricle’s walls thicken and left ventricle dialates (Galanti et al., 2016; Weeks and McMullen, 2011).

Athlete’s heart is considered reversible, but studies of retired athletes show that even years into retirement, the heart does not completely regress to normal physiology (O’Keefe et al., 2012). Also, new studies are beginning to show that athletes who undergo chronic high-intensity and high endurance exercise training may exhibit cardiac remodeling (that is not completely benign), increased vagal and sympathetic tones, bradycardia, inflammatory changes, myocardial fibrosis and atrial dialation (O’Keefe et al., 2012).

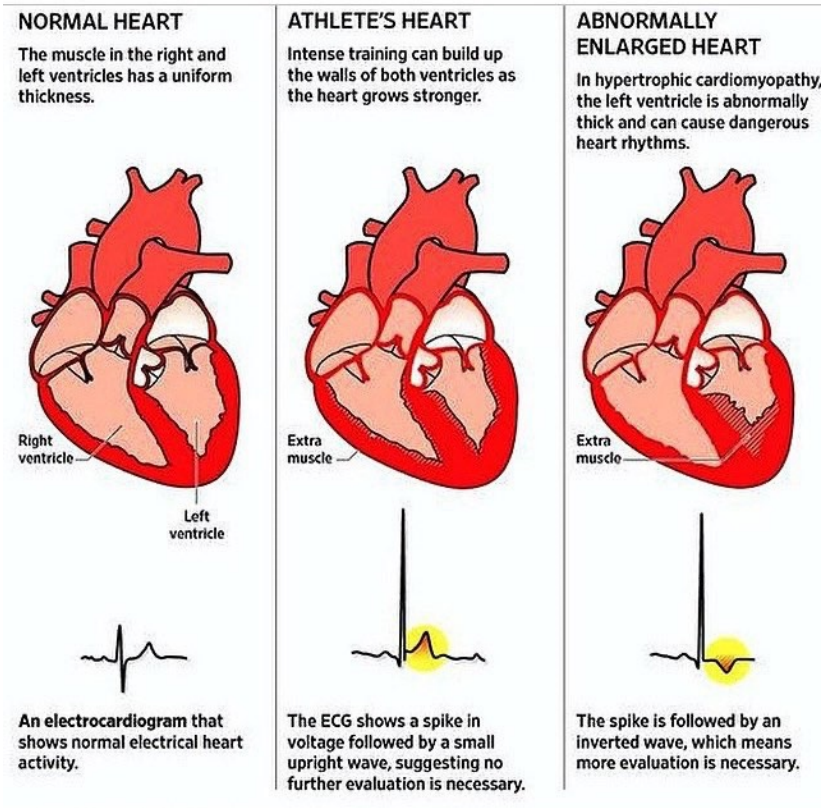


Figure 20. Normal heart vs athlete's heart vs pathological heart. Retrieved from Medical doctor (https://www.picluck.net/media/947934150023816202_1591812152).

The most evident changes found during exercise are:

- Acute dilation of the right atrium
- Acute dilation of the right ventricle
- Sudden decrease in right ventricular ejection fraction (EF)
- Elevated troponin and B-type natriuretic peptides
- Myocardial fibrosis
- Aortic valve stiffness
- Higher than expected levels of coronary artery calcium and calcified coronary plaque volume.

Another consequence of the endurance training is the considerable remodeling of the vascular system, especially in the skeletal muscles subjected to the exercise, including an increase in the diameter of large conducting vessels like the femoral artery, in the number of arterioles and in the capillary density. This type of training

promotes volume hypertrophy that also involves de novo cardiomyocyte formation (Hawley et al., 2014). More research in recreational athletes finishing a marathon has found changes in cardiac dysfunction and injury, strongly influenced by the level of preparation undertaken. The majority of the most marked abnormalities in cardiac structure or function, as well as cardiac biomarker changes, were seen in those athletes training less than 35 miles/week before the marathon (Neilan et al., 2006).

On the other hand, it has been shown that intense races such as marathons and ultramarathons in most of the runners, reduce cardiac function temporarily once the race has ended. But this reduction does not imply permanent damage to the heart and returns to the baseline within a week. Anyway, physicians are worried about the immediate post-marathon elevation of enzymes such as cardiac troponin that are normally released during a heart attack. However, these biomarkers quickly return to their normal reference level during 24 h after a marathon, while in a myocardial infarction they remain elevated for a week. These biomarkers elevations are considered inherent and expected with exercise as a sign of cardiac stress or simply due to the increase in acidity or temperature of the blood, and in any case, it has been considered to be a sign of permanent cardiac damage or negative health (George et al., 2012). In Chapter 2 a noticeable increase of 3 cardiac biomarkers after a marathon is assessed and how it affects the preparation and participation to the race.

3.3 Sudden cardiac death in athletes

Physical inactivity increases the risk of developing a variety of diseases, including obesity, diabetes, hypertension, osteoporosis, or depression. Most of the benefits that regular exercise contributes are shown in Figure 2. In addition, studies based on nearly 900,000 participants, demonstrated that physical activity is associated with a marked decrease in cardiovascular and all-cause mortality, with risk reductions of 30–50% for cardiovascular mortality and 20–50% for all-cause mortality (Nocon et al., 2008). Episodes of vigorous exercise (as explained in 2.), such as marathon running may precipitate acute coronary syndrome (ACS) and sudden death in 1 per 50,000 marathon participants (Siegel et al., 2008). Without an underlying heart condition or disease, the risk of sudden cardiac death during a race is extremely low.

There is only a slightly increased risk of a cardiac arrest during strenuous exercise if the runner is predisposed. While most deaths may be attributed to inherited cardiomyopathies and channelopathies in those younger than 35 years old, there is no absolute cutoff. Thus, athletes in their thirties and forties (the median age in many competitive sports) are at greatest risk of sudden cardiac death caused by inherited and acquired causes (Figure 21), as is the coronary atherosclerotic disease that begins to express itself at this age (La Gerche et al., 2013). Cardiac arrest can be caused by a lack of blood flow to the heart due to coronary-artery disease, or by a structural abnormality of the heart that causes it to suddenly start beating irregularly (an arrhythmia) and ineffectively (Kim et al., 2012). These situations are more likely to occur when the heart pumps 5 times more blood during exercise. Sudden cardiac death caused by either a “heart attack” or an arrhythmia can sometimes be reversed with cardiopulmonary resuscitation (CPR), especially if it started quickly after the collapse and a defibrillator is present. Some cases in races I have done were in Comrades Marathon in 2007 with 2 men over 25,000 runners, in Marathon des Sables in different years with 4 men over 20,000 participants, and less prevalent cases in Cursa dels Bombers, Mitja marató de Gavà, Behobia Sant Sebastian, Triatló Berga, Garmin Barcelona Triatló among others.

Intense physical exercise, in people with structural heart disease, increases cardiovascular risk during or in the hour after exercise and the most relevant complications are sudden death and acute myocardial infarction (AMI) (Mons et al., 2014). On the other hand, athletes most prone to atrial fibrillation (AF) are those males, in their 4th-5th decade of life, who have exercised resistance at high-intensity for more than 10 years (Drca et al., 2014). Another study with older men and women (mean age 73 years) reported that exercise intensity showed the familiar reverse J-shaped relationship with the risk of atrial fibrillation (AF) (Figure 22) (Mozaffarian et al., 2008), and veteran male endurance runners (who had run at least 1 marathon each year for 25 consecutive years) had significantly increased amounts of coronary plaque, both hard and soft (Figure 23) (O’Keefe et al., 2014; Schwartz et al., 2014).

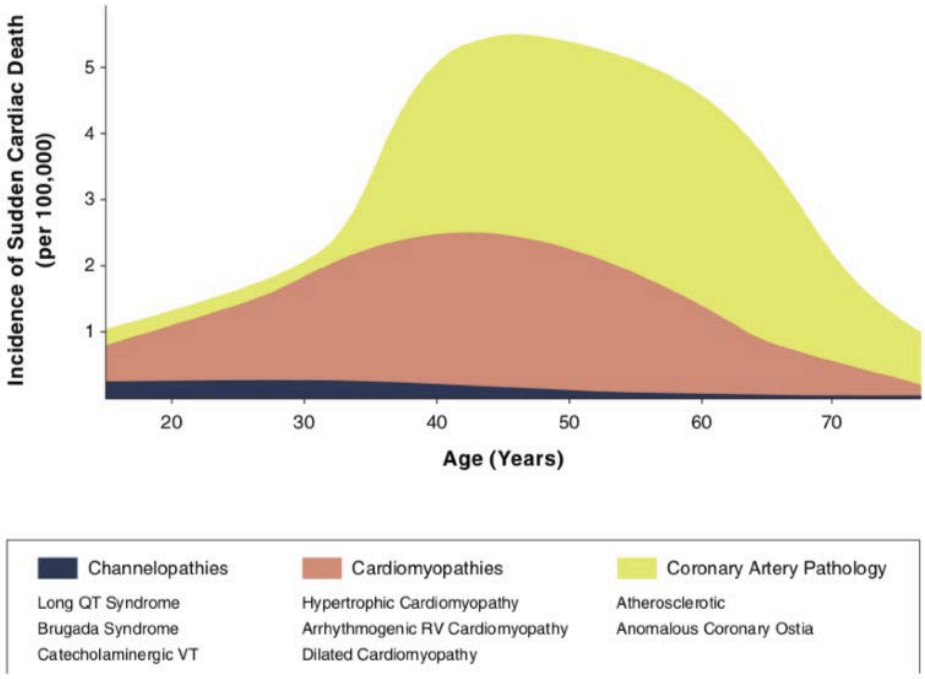


Figure 21. Age-dependent changes in incidence and etiology of sudden cardiac death. RV, right ventricular; VT, ventricular tachycardia. Retrieved from La Gerche et al. (2013).

Male marathon participants are associated with a low overall risk of cardiac arrest and sudden death, but the incidence rate has increased during the past decade. In Figure 24 the cardiac arrest causes from 59 runners where 71% died are illustrated. The incidence rates of cardiac arrest and sudden death during long-distance running races from 2000 to 2010, were 0.54 per 100,000 (Kim et al., 2012), better rates than the ones from Siegel et al. (2008) or from the systematic review done by Waite et al. (2016), where the incidence of sudden cardiac death rate in marathoners was 0.6-1.9 per 100.000 runners (34-year period involving nearly 4 million runners).

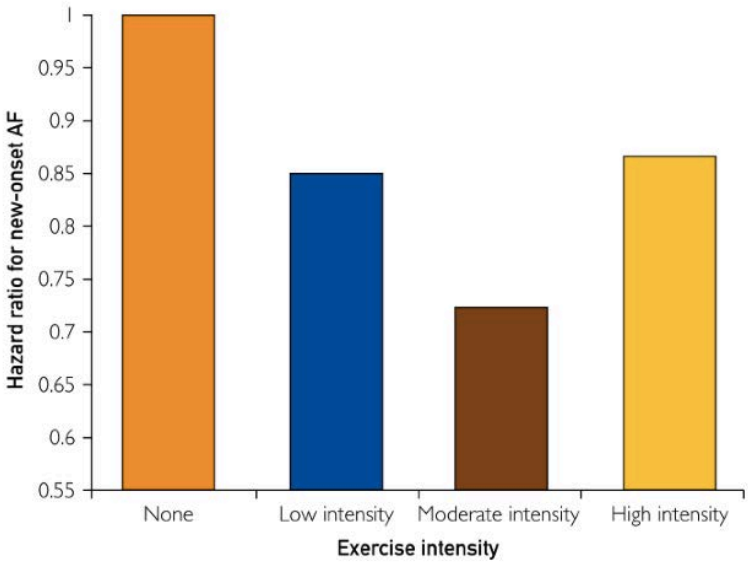


Figure 22. Risk of new-onset atrial fibrillation (AF) in older adults depending on exercise intensity. Retrieved from O'Keefe et al. (2014) and adapted from Mozzafarian et al. (2008).

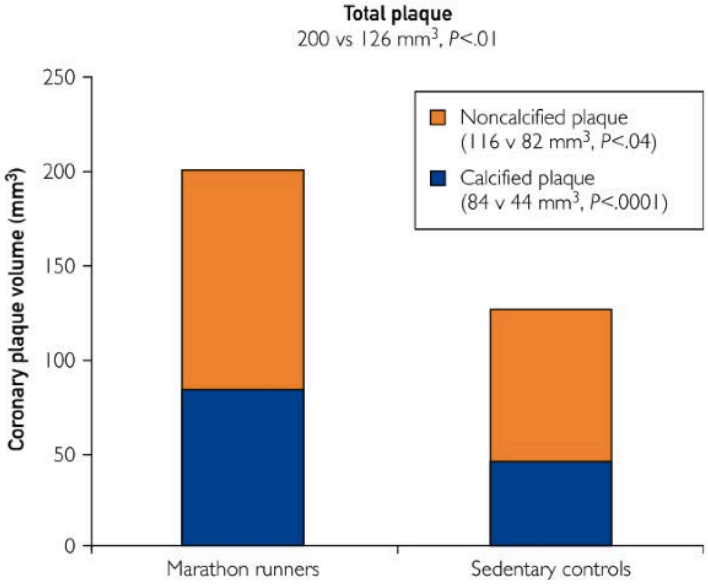


Figure 23. Total coronary plaque volume (noncalcified plaque volume and calcified plaque volume) in male marathoners compared to sedentary controls. Retrieved from O'Keefe et al. (2014) and adapted from Schwartz et al. (2014).

Another study done in rats (with heart rates of 500 beats/min vs 60 beats/min in humans) (Benito et al., 2011), which ran at high-intensity during different periods of 4, 8 and 16 weeks, the rats that performed more exercise compared to sedentary

ones, developed eccentric hypertrophy and diastolic dysfunction, atrial dilation, and collagen deposition in the right ventricle. In 42% of the marathon rats, a ventricular tachycardia could be induced. The damage observed in the myocyte may be associated with inflammation and subsequent myocardial fibrosis, resulting in a substrate for the future appearance of arrhythmias or cardiac dysfunction. But all the fibrotic changes caused by the 16 weeks of intensive exercise reversed after 8 weeks of rest. Although it would be desirable to replicate this research in humans (if it can be done ethically), this study supports the hypothesis that running repeatedly at high-intensity causes an unfavorable cardiac remodeling that can induce arrhythmias.

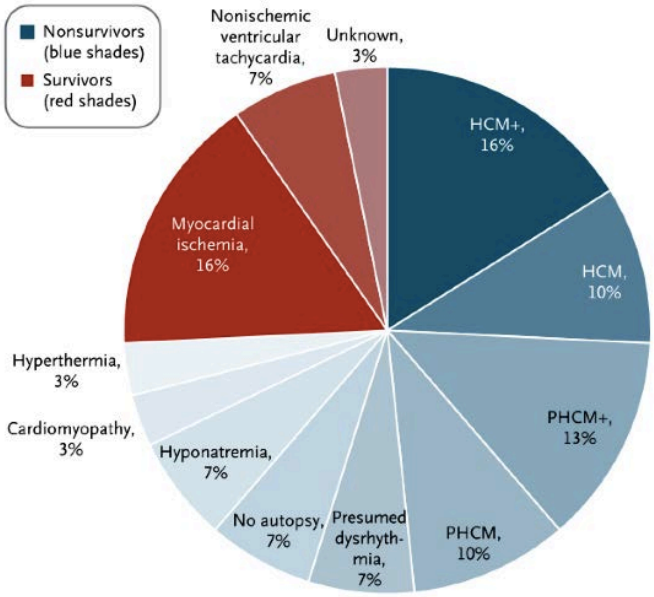


Figure 24. Causes of cardiac arrest in 59 runners (42±13 years) over 10.9 million analyzed (HCM: hypertrophic cardiomyopathy, PHCM: possible hypertrophic cardiomyopathy). Retrieved from Kim et al. (2012).

Another consequence during physical exercise at moderate to high-intensity can be ischemia. Perhaps it is not prolonged enough to produce irreversible myocardial injury but might be sufficient for shedding on the surface of the plasma membrane cardiac myocytes blebs (like ‘’bubbles’’). These bubbles would then be released into the circulation along with their cytoplasmic content. But when ischemia is prolonged, the blebs grow, collapse, and produce cell necrosis (Brancaccio et al., 2010).

On the other hand, before studying the Barcelona marathon, it was assessed in ultra marathoners if the endurance training was associated with marked increases in cardiac output requirements. We wanted to know if this workload imposed a high degree of stress on all myocardial structures, especially in the right ventricle which typically works at low pressures under physiological conditions. An acute right ventricle impairment was found, dilatation and decreased deformation, although a high individual variability was present. Also, different patterns of right ventricle adaptation were identified, independent of dehydration or previous training. And finally, it was concluded that a dynamic evaluation of the right ventricle response (echocardiographic analysis) could help to identify which runners have a better or worse right ventricle adaptation to exercise and avoid long-term consequences (Sanz et al., 2015 and 2016).

Thereby, how can heart health be controlled biochemically after intense and long exercise like a marathon? Will the increase in circulating concentrations of cardiac biomarkers be sufficient? It can be, but never a single biomarker will be enough to evaluate how a marathon can be dangerous to our heart. That is why, in this thesis, several cardiac biomarkers are analyzed to evaluate which is the impact of a marathon on the cardiovascular system.

3.4 Cardiac blood biomarkers

Heart failure is a major and growing public health problem and begins with myocardial injury, usually secondary to myocardial infarction, ischemia, hypertension, or other etiologies like genetic, neurohormonal, inflammatory, and biochemical changes (Braunwald, 2008). Seven classes of biomarkers have been described as a multi-marker strategy to define risk stratification for heart failure (Figure 25). It has been demonstrated that using troponin together with brain-natriuretic peptide (BNP) can achieve a more accurate stratification of risk than can be obtained with either biomarker alone. The accuracy of risk prediction has also been enhanced when a natriuretic peptide has been coupled with other biomarkers of myocardial stress like ST2, as well as with inflammatory biomarkers like C-reactive protein (CRP) (Braunwald, 2008).

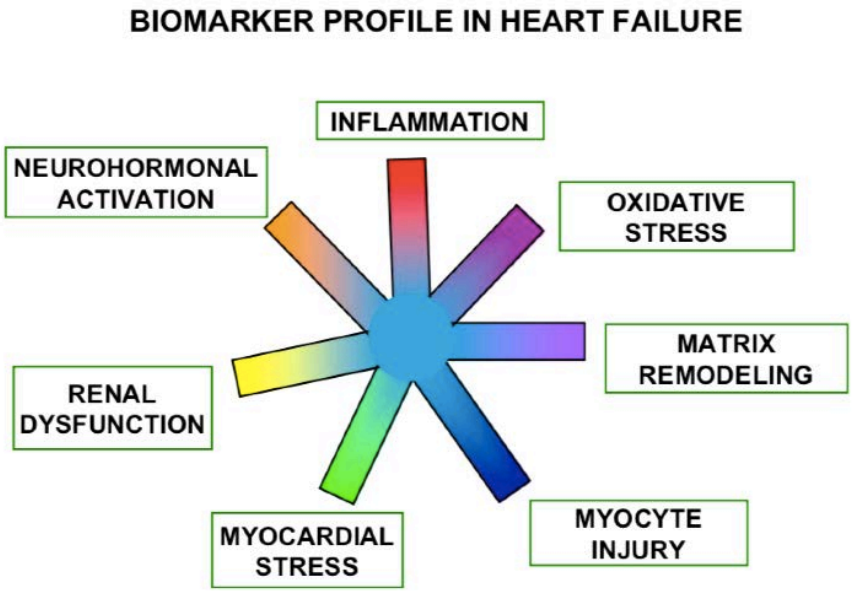


Figure 25. Seven axes profile representing biomarkers of each class. A resultant biomarker profile should enhance prevention, treatment and prognostication. Retrieved from Braunwald (2009).

Biomarkers in heart failure can be classified into 7 main groups: inflammation, oxidative stress, extracellular-matrix remodeling, neurohormones, myocyte injury, myocyte stress and new biomarkers. In the Barcelona marathon participants, inflammation (C-reactive protein), myocyte injury (Hs-TnT), and stress (NT-proBNP and ST2) biomarkers were assessed.

One of the things that protects against cardiac biomarkers elevations (with echocardiographic evidence of cardiac dysfunction) while doing endurance exercise, it is an appropriate individualized preparation/training (Neilan et al., 2006). And precisely how affects the preparation prior to the marathon and the race itself on cardiovascular health is what is evaluated in this thesis.

3.4.1 High-sensitivity troponin T (Hs-cTnT)

Troponins are proteins that work to regulate muscle contraction, making actin-myosin interactions sensitive to cytosolic calcium levels. The troponin complex with the tropomyosin and 7 actin monomers, constitute the myocardial sarcomeric unit (see Figure 11). The troponin complex is composed of 3 subunits: troponin T (TnT) (37 kDa), which anchors the complex to the tropomyosin strand of the thin filament; troponin C (TnC) (18 kDa), which binds calcium ions released from the sarcoplasmic reticulum; and troponin I (TnI) (22.5 kDa), which inhibits the enzymatic hydrolysis of adenosine triphosphate that powers muscle contraction (Shave et al., 2010) (Figure 26). Different forms of TnT and TnI are found in cardiac and skeletal muscle with cardiac (c) and skeletal (s) isoforms of TnT (cTnT, sTnT) and TnI (cTnI, sTnI) each encoded by separate genes. The sensitivity and specificity of cTnT and cTnI for the detection of myocardial injury are superior to older biomarkers, including LDH, CK, CK-MB, and myoglobin. Troponin tests have improved over the past 10 years, since 2012 cTn high-sensitivity (hs) assays, which have the ability to reliably detect very low troponin levels in plasma (enabling the early detection of troponin release and allowing an earlier diagnosis), and have a much lower absolute coefficient of variation (enabling the reliable detection of small variations over the time and differentiates chronic elevations from those acute) (Costabel et al., 2017). In this thesis, we have used Hs-cTnT because of its sensitivity and because it is the preferred biomarker in the diagnostic of myocardial infarctions.

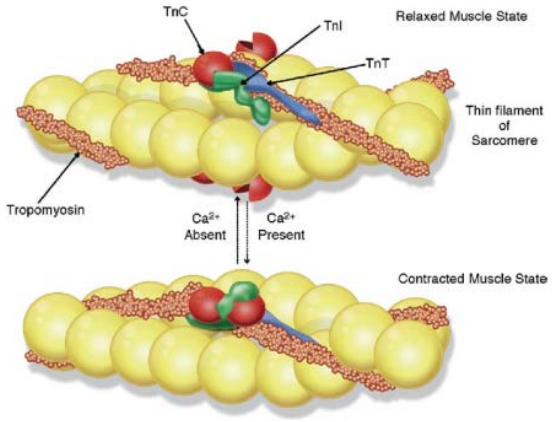


Figure 26. Scheme of the cardiac muscle where the 3 troponin isoforms can be located in relation to actin and tropomyosin. Retrieved from Shave et al. (2010).

High-sensitivity troponin T is the **myocyte stress/injury biomarker**. Its elevation is associated with an incomplete myocardial adaptation to exercise (Gresslien and Agewall, 2016). The post-exercise cardiac troponin increase is related to the training status and it is greater in the less trained marathoners (Mehta et al., 2012). Also, this increase is directly related to the intensity (Brancaccio et al., 2010; Richardson et al., 2018) and the duration of time spent participating in a marathon (Schmied, 2014).

The release of hs-cTnT is likely multifactorial and it can also depend on subendocardial ischemia due to wall stress, myocardial apoptosis, or oxidative injury. When a correlation between echocardiograms and blood biomarkers has been done in recreational athletes completing a marathon, there is evidence of cardiac dysfunction and injury. With prolonged exercise, there is an increased pulmonary artery pressure, increased right ventricle dimensions, and decreased right ventricle function, all of which correlate with the release of cTnT. This exertion also provokes alterations in left ventricle diastolic function, which correlates with an increase in another biomarker called NT-proBNP (see 3.4.2). All these changes are strongly influenced by the level of training undertaken by these amateur athletes, such that the majority of the most marked abnormalities in cardiac structure or function, as well as cardiac biomarker changes, are seen in those athletes training less than 35 miles/wk before the marathon (Neilan et al., 2006).

Normal values of hs-cTnT are under 14 ng/L. A meta-analysis made with 16 studies of 939 marathon runners showed high levels of troponin T after the race (Regwan et al., 2010). The majority of the studies of troponin and marathon running have revealed increased levels in between 0 and 100% of subjects, but multiple factors can influence the post-exercise levels of troponin (especially intensity, age, training experience, time of sampling and type of essay) (Gresslien and Agewall, 2016).

Even now, the cardiac troponin level increase in marathoners is not understood, and it will be necessary to cross these values with other biomarkers, not only cardiac, to draw conclusions.

3.4.2 Amino-terminal pro-B type natriuretic peptide (NT-proBNP)

During an episode of hemodynamic stress, when the ventricles are dilated, hypertrophic, or subject to increased wall tension, a pro hormone BNP is cleaved by a circulating endoprotease into two polypeptides; the inactive NT-pro-BNP of 76 amino acids (with a longer half-life), and the bioactive peptide BNP of 32 amino acids. BNP causes actions that oppose the physiological abnormalities in heart failure, like arterial vasodilation, diuresis, and natriuresis, and reduces the activities of the renin–angiotensin–aldosterone system and the sympathetic nervous system (Braunwald, 2008) (Figure 27). The natriuretic peptides are cleared by the kidneys, and the hypervolemia and hypertension characteristic of renal failure enhance the secretion and elevate the levels of BNP, especially the NT-pro-BNP (Daniels and Maisel, 2007). There are three major natriuretic peptides that share a common 17-amino-acid ring structure; atrial (A-type) natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are of myocardial cell origin, and C-type natriuretic peptide (CNP) is of endothelial origin. All three peptides are secreted in an attempt to correct the vasoconstrictive, retain sodium, antidiuretic, and antifibrotic effects caused by the neurohormonal imbalance in heart failure (Bhalla et al., 2004).

Both BNP and NT-proBNP levels in the blood are used for screening and diagnosing acute congestive heart failure and may be useful to establish prognosis in heart failure, as both markers are typically higher in patients with worse outcomes. Both are **myocyte stress biomarkers.**

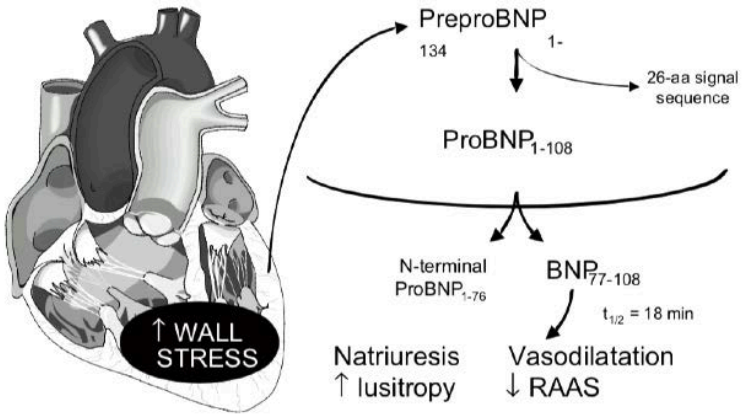


Figure 27. The structure of proBNP, BNP, and N-terminal proBNP (BNP=B-type natriuretic peptide; RAAS=renin-angiotensin-aldosterone system). Retrieved from Bhalla et al. (2004).

The cutoff value for NT-proBNP is 94 ng/L and a value higher than 125 ng/L is considered clinically relevant and an indicator of cardiac damage risk (Koç et al., 2009). In the context of marathon running studies, NT-proBNP is reported to be elevated after the race with concentrations of 183 and 137 ng/L. Other authors also reported that 38 of their 46 subjects recorded concentrations that were comparable with those indicating cardiac events and disease. Similarly, another study that measured proBNP following repeated bouts of severe prolonged running, reported that while some runners displayed elevated concentrations of pro-BNP >100 ng/L above pre-exercise concentrations (>200% increase), these did not exceed the clinical cut off (>125 ng/L) for congestive heart failure patients, and had returned to pre-exercise concentrations within 24 h of the last exercise. So, despite the temporary elevations in these biomarkers, it has been shown how severe prolonged running has a minimal clinical impact (Bird et al., 2014).

Other studies of changes in cardiac troponin T and I and B-type natriuretic peptide (BNP and NT-proBNP) levels in asymptomatic runners who completed a marathon have demonstrated that mild to moderate elevations are common without apparent acute coronary syndrome or heart failure. However, even though the mechanism of cardiac injury and myocyte stress (reflected by elevation of troponin and B-type natriuretic peptide concentrations respectively) remains under debate, presumably these elevations result from an incomplete myocardial adaptation to training in which vulnerable myocytes are selectively eliminated, particularly in athletes with lesser training (Siegel et al., 2008).

3.4.3 Suppression of tumorigenicity 2 (ST2)

Beyond the natriuretic peptides, there is increasing interest in a class of markers that predict deleterious processes subsequent to ventricular remodeling (wall stretch) like ST2. When cultured monocytes are subjected to mechanical strain, the protein ST2 is secreted. It is a member of the interleukin-1 receptor family. Infusion of soluble ST2 appears to dampen inflammatory responses by suppressing the production of the inflammatory cytokines interleukin-6 and interleukin-12.

ST2 is an **extracellular matrix remodeling and fibrosis, inflammation and myocardial strain biomarker**.

Despite the well-documented successes and strengths of natriuretic peptides, there is room for improvement in the evaluation and risk stratification of patients with HF or at risk for HF. One of the biomarkers to fill this gap is ST2. As ST2 reflects inflammation, fibrosis, and cardiac strain, it can be an independent predictor in an inflammatory status as secondary to HF and renal dysfunction. Also, ST2 is valuable for predicting sudden cardiac death in ambulatory patients with mild to moderate chronic HF and left ventricular systolic dysfunction, and is a strong prognostic marker for short-term mortality risk (Bayés-Genis et al., 2015). In patients with acute myocardial infarction, elevated soluble ST2 (sST2) levels are associated with an increased risk of mortality or HF, independent of natriuretic peptides (Shah and Januzzi, 2010). And this elevated level of ST2 in patients with severe HF are the result of cellular death, tissue fibrosis, reduced cardiac function, and an increase in the rate of disease progression (Braunwald, 2008).

Individuals are at a higher risk of adverse outcomes when ST2 levels are above a cut-off value of 35 ng/mL (Weinberg et al., 2003). No studies have been done with marathoners and ST2 save the one presented in this thesis, where levels of ST2 before and 48 h post-marathon were elevated in nearly 50% of the runners, and just finishing the ST2 levels were over the cut-off in nearly 90% of the runners. Recently it has been published in another study related to marathon and ST2 and found increased ST2 concentrations in amateur marathoners with ST2 concentrations exceeding cutoff values both at baseline (48%) and finish (94%) and found higher ST2 concentrations in faster runners (Aengevaeren et al., 2019).

Far from supplanting natriuretic peptides and sensitive troponin assays, novel markers such as ST2 will be complementary and powerful predictors of cardiovascular risk. There is substantial experience with measuring the natriuretic peptides like NT-proBNP (for which excellent assays are available) or troponins, but less information is available for ST2 and analytic methods for determining ST2 have not yet been standardized (Braunwald, 2008). That’s why analyzing ST2 in marathoners is pioneering and can contribute to the other biomarkers evaluated in order to present an excellent risk profile related to cardiovascular events in runners.

4. Endurance exercise and immunity

Plenty of hostile bacteria, fungi, and viruses live around us, but we remain healthy most of the time thanks to the two intrinsic defense systems that act both independently and cooperatively to provide immunity: the innate/nonspecific system and the adaptative/ specific defense system (Figure 28). The first one responds within minutes with the “first line of defense” (external body membranes as skin and mucosa) and “the second line of defense” (antimicrobial proteins and phagocytes among others triggering inflammation). The second one attacks particular foreign substances and is the “third line of defense”. Our body is protected from most infectious microorganisms, cancer cells, and transplanted organs or grafts, thanks to a direct (cell attack) or indirect (releasing mobilizing chemicals and protective antibody molecules) actions (Marieb, 2004).

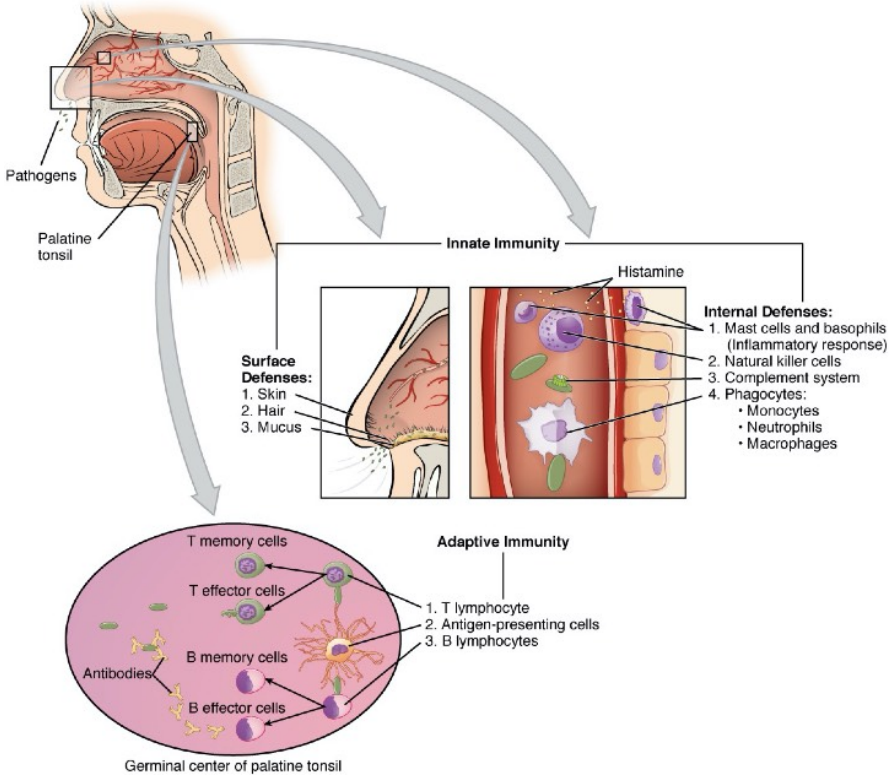


Figure 28. Cooperation between Innate and Adaptive Immune Responses being more effective. Retrieved from Betts et al. and OpenStax, Anatomy and Physiology: The Lymphatic and Immune System (2017).

Heavy exertion or exercise lowers resistance and is a predisposing factor to respiratory tract infections (RI). However, at the same time, regular exercise confers resistance against infection. The relationship between exercise and RI may be modeled as a "J" curve (Figure 29). This model suggests that while doing moderate exercise the RI risk decreases below of a sedentary individual, but doing high-intensity exercise the RI risk is the highest. Several epidemiological reports suggest that athletes engaging in marathon-type events and/or very heavy training are at increased risk of RI (lower distances even have a slight reduction of RI incidence). The risk appears to be especially high during the 1- or 2-week period after the marathon (Nieman, 1997). But the acute decrease in immune function is transient and is dependent on the exercise intensity (as shown in Figure 29) and duration. Repeated activity with insufficient recovery, such as during heavy periods of training and competition, appears to exacerbate the situation leading to a chronic depression of several aspects of immune function (Bishop and Gleeson, 2009).

Research in more than 2300 marathoners has shown that training more than 96 km/ week doubled their odds for sickness compared with those training less than 32 km/ week. Other studies in ultra runners have seen how the risk of infection increased 100% to 500% and around 25% of finishers reported respiratory symptoms (Gill et al., 2013 and 2014).

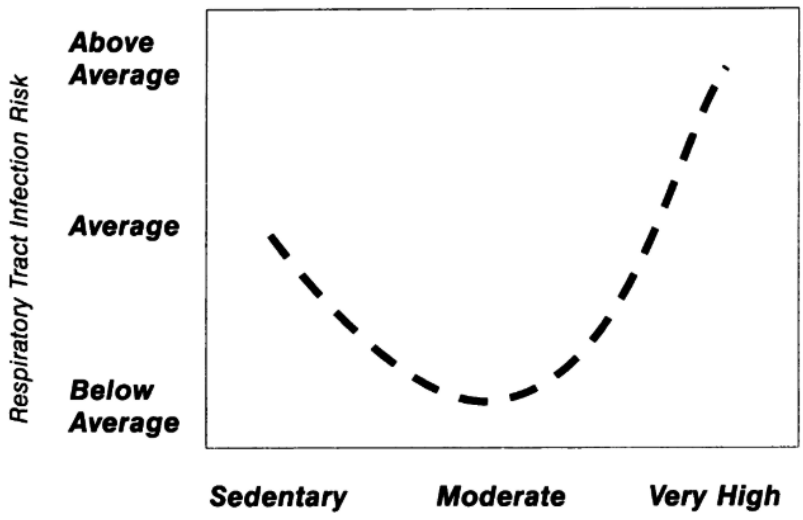


Figure 29. "J" shaped model suggesting that moderate exercise may lower risk of respiratory infections while very high-intensity exercise may increase the risk. Retrieved from Nieman (1997).

When an increased risk of RI appears, it means that the immune function is negatively affected. Many components of the immune system change after prolonged exercise (Nieman, 2000), some of these changes are:

- High neutrophil and low lymphocyte blood count induced by high plasma cortisol (the immune system is suppressed and stressed, decreasing host protection against viruses and bacteria).
- Increase in blood granulocyte and monocyte phagocytosis, but a decrease in nasal neutrophil phagocytosis (substances released from injured muscle cells initiate an inflammatory response).
- Decrease in nasal and salivary IgA concentration (the major effector of host defense of the upper respiratory tract is reduced).
- Decrease in nasal mucociliary clearance.
- Decrease in granulocyte and macrophage oxidative burst activity (killing activity).
- Decrease in natural killer (NK) cell cytotoxic activity (then the protection against viruses and bacteria is reduced).
- Decrease in mitogen-induced lymphocyte proliferation (T-cell function is decreased).
- Decrease in the delayed-type hypersensitivity skin response.
- Increase in plasma concentrations of pro- and anti-inflammatory cytokines and chemokines.
- Blunted major histocompatibility complex II expression and antigen presentation in macrophages.

In response to a marathon, natural killer cells, neutrophils, and macrophages (of the innate immune system) exhibit the greatest changes in terms of numbers and function, of all immune cells. During this ‘open window’ of altered immunity (which may last between 3 and 72 hours), viruses and bacteria may gain a foothold, increasing the risk of infection (Figure 30). It is necessary to define if athletes with the most extreme immune suppression following heavy exertion are those that contract an infection during the following 1–2 weeks (Nieman, 2007).

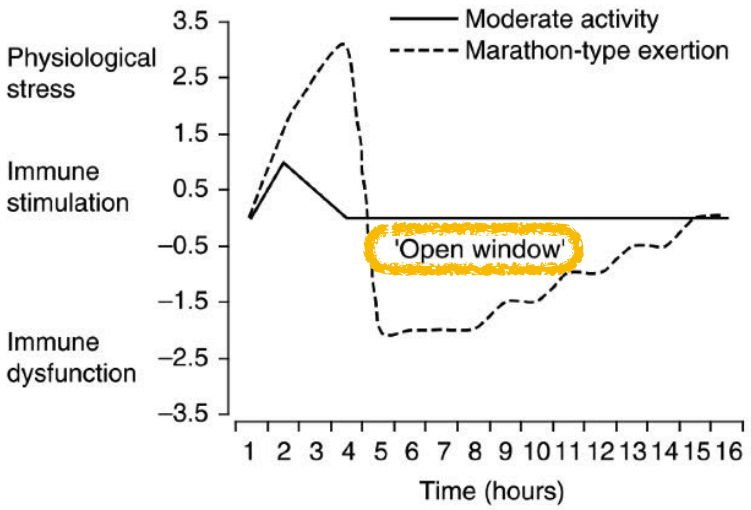


Figure 30. The 'open window' theory. Moderate exercise causes mild immune changes while marathon-type exercise leads to immune dysfunction that increases the likelihood for opportunistic upper respiratory tract infections. Retrieved from Nieman (2007).

In addition, when there is muscle tissue damage, physiological and immune responses are activated. Inflammation can be tracked indirectly through key components of the inflammation process that can enter into systemic blood circulation. Chronic inflammation that persists after damage indicates injury or stress from overtraining, infection, autoimmune disease, cardiovascular disease, or other major health concerns. In any case, chronic inflammation is a positive feedback phenomenon that can impact the athlete's health and performance. When markers of muscle damage are released into circulation, immune cells migrate to the site of tissue injury and differentiate into mature pro-inflammatory macrophages. These macrophages also release a number of growth factors, cytokines, and other signaling molecules that promote the inflammatory process by recruiting other cells required for skeletal muscle regeneration. During inflammation, liver tissue may be stimulated to produce an acute-phase response that recruits vascular tissue activation, systemic immune response, and endocrine function among others (Lee et al., 2017).

Interactions between muscle and inflammatory cells are important in determining the course of muscle injury and remodeling. Macrophages can injure muscle cells in vitro and in vivo and their cytolytic capacity is increased by the presence of

neutrophils. Cytotoxicity assays have shown that macrophages lyse target muscle cells by nitric oxide (NO)-dependent and superoxide-independent mechanism. Experimental observations support the potential interactions between neutrophils, macrophages, and muscle with a potential role in promoting injury or repair (Figure 31) (Tidball, 2005).

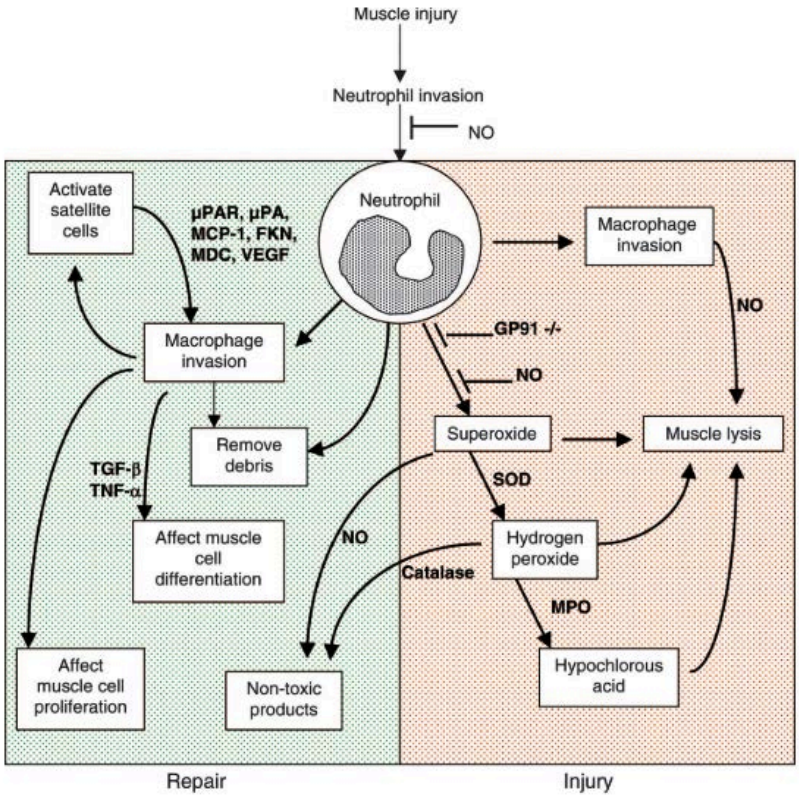


Figure 31. Schematic of potential mediators of inflammatory cell interactions with injured muscle (NO: nitric oxide; μPAR: urokinase type plasminogen-activator receptor; μPA, urokinase; MCP-1: monocyte chemoattractant protein-1; FKN:fractalkine; MDC: macrophage-derived chemokine; VEGF: vascular endothelial growth factor; SOD: superoxide dismutase; MPO:myeloperoxidase). Retrieved from Tidball (2005).

A physical demand, such as a marathon, increases the migration of white blood cells to the sites of muscle injury, and induces acute-phase inflammatory reactions, producing a large number of acute-phase proteins and cytokines related to innate immunity such as secretory Immunoglobulin A (sIgA) or inflammatory chemokines (Gleeson and Bishop, 2013; Trochimiak and Hubner-Wozniak, 2012).

4.1 Salivary immunity

The Common Mucosal Immune System is a network of organized structures, such as the respiratory system and oral cavity with the nasal and bronchial/tracheal lymphoid tissue, and salivary glands (Bishop and Gleeson, 2009).

Saliva, a mucosal secretion regulated by the autonomic nervous system that contains a significant amount of secretory antibodies, can be influenced by strenuous exercise like a marathon. Due to recent advances in the measurement of antimicrobial peptides and immunoglobulins in saliva, the study of mucosal immunity-related with exercise has increased.

Saliva is produced by three pairs of major salivary glands—the parotid, submandibular, and sublingual; in addition to many smaller glands found in the submucosa under most soft tissue surfaces of the mouth. We produce around 1500 mL of saliva each day. Whereas IgA is transported into saliva across salivary glandular epithelial cells, lactoferrin is synthesized and secreted by acinar cells in the secretory endpiece of salivary glands and lysozyme are the macrophages in the oral mucosa. Following secretion, both lysozyme and lactoferrin require pepsin digestion for activation into their anti-microbial form (Bishop and Gleeson, 2009). Fluid balance studies in athletes have observed significant reductions in salivary flow rate when hypohydration exists with different results in sIgA and Lysozyme secretion rates (Fortes et al., 2012; Gill et al., 2013).

RI is protected by an array of salivary antimicrobial proteins (sAMPs) that act as the first line of defense against invading microorganisms. sAMPs range from small cationic peptides to larger polypeptides and proteins that are typically secreted by phagocytes, epithelial cells, and salivary glands. Exercise-induced changes in sAMPs depend on the fitness level, due to training-induced alterations in parasympathetic and sympathetic nervous system activation (Kunz et al., 2015). The most common antibody on the mucosal surface, sIgA, suffers a decrease after long bouts of exercise (more than 90 minutes), related with a high incidence of RI (Nieman et al., 2002). Other studies suggest that immune factors present in mucosal secretions, including AMPs and immune mediators such as chemokines, also change after prolonged running, like salivary lactoferrin (one of the most abundant AMPs) that increases after a 50 km ultramarathon (Ihalainen et al., 2016).

However, limited data regarding sIgA and other AMPs, chemokines, and the presence of lower respiratory tract infections in marathon runners exist. In this thesis, the impact of a marathon on salivary immunity and respiratory tract infections is analyzed.

4.2 Saliva biomarkers

4.2.1 Antimicrobial peptides: sIgA, lysozyme and lactoferrin

Secretory immunoglobulin A (sIgA) is the primary immunoglobulin in saliva that functions as part of the body's initial defense against invading microbes. This dimeric molecule is formed by two IgA monomers joined by the J-chain and covalently bound to a secretory component, which is produced in plasma cells that lie adjacent to salivary glands, from which it is excreted in a saliva flow rate-dependent fashion. Each IgA monomer comprises two heavy chains and two light chains, joined by disulfide bonds (Figure 32). While sIgA is the most abundant antimicrobial protein found in mucous secretions including saliva; additionally, α-amylase, lactoferrin and lysozyme provide the first line of defense against pathogens that might be present on mucosal surfaces (Bishop and Gleeson, 2009; Walsh et al., 2011).

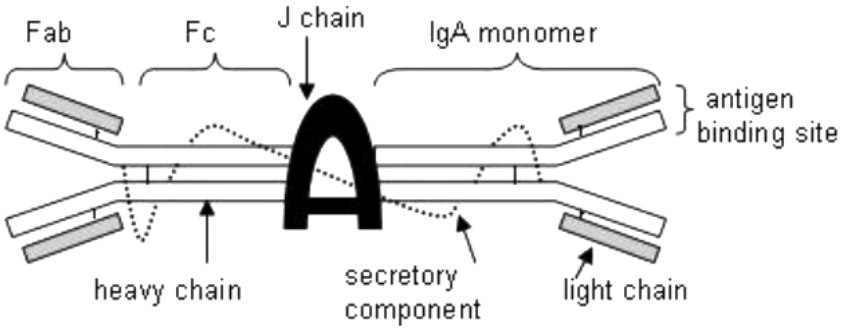


Figure 32. Schematic representation of sIgA (Fab: fragment antibody binding portion, Fc: fragment crystallizable portion). Retrieved from Bishop and Gleeson (2009).

Marathon and endurance performance have been shown to negatively affect sIgA, and additionally, ultra-endurance events resulted in declines in immune defense in the oral cavity, pulmonary function, and salivary flow rate (Bellar et al., 2017). Only prolonged exercise and intensified training (but not moderate exercise) can evoke decreases in sIgA. Acute exercise altered mucosal immunity is related to the activation of the sympathetic nervous system and its associated effects on salivary protein exocytosis (a form of active transport and bulk transport in which a cell transports molecules out of it by expelling them) and sIgA transcytosis (a type of transcellular transport in which various macromolecules are transported across the interior of a cell) (Walsh et al., 2011).

Lysozyme (Lys) and lactoferrin (Lac) are the two most abundant AMPs. Salivary Lys and Lac are produced by epithelial cells, salivary glands, and granules of neutrophils. Lys and Lac may function synergistically to augment immunity, but each one has a specific function; Lys enhances protection against gram-positive bacteria and Lac improves immunity by inhibiting iron uptake by microorganisms and thereby reducing bacterial growth (Gillum et al., 2013). One study assessing the effect of a short run (45 minutes) instead of a long one, resulted in increased Lac concentration which could lead to a decrease in inflammation. These results add evidence to the anti-inflammatory effects of moderate exercise. Conversely, a decrease seen in intracellular Lys content could be the cause of increased Lys in exocrine solutions (Gillum et al., 2017).

A reliable marker of hypothalamic-pituitary-adrenal axis activity is the cortisol hormone, segregated in response to intense exercise to aid in the metabolism of fat, protein, and carbohydrates. It has been seen how cortisol can alter mucosal immunity through a reduction in sIgA and lysozyme. Also after analyzing 14 runners in a 50 km race, IgA was more sensitive to prolonged running than either Lys or Lac, of, which secretion rates were unaffected (Gillum et al., 2013).

4.2.2 Chemokines: Groα, Groβ, MCP-1

Cytokines derived predominantly from mononuclear phagocytic cells are uniquely important in innate immunity and both initiate immune responses and generate symptoms associated with infections and inflammatory disorders. Cytokines are signaling molecules produced by cells for specific biological functions (interleukin for example is a type of cytokine produced by white cells). Cytokine is a general term used for all signaling molecules while chemokines (chemoattractant molecules) are specific cytokines that function by attracting cells to sites of infection/inflammation (Borish and Steinke, 2003).

Chemokines have demonstrated an important role during exercise in the regulation of local immunity against infections by contributing to the tissue infiltration of leukocytes and in the attraction of neutrophils to the site of inflammation. Also, several inflammatory chemokines have demonstrated a potential role in the regulation of immune response during exercise (Terra et al., 2012).

Some of these chemokines are:

- Growth-Regulated Oncogene-alpha (Groα or CXCL1): neutrophil-activating protein and melanoma growth stimulating activity. It is found to be an inflammatory factor that plays a critical role in wound healing by modulating cell migration and angiogenesis.
- Growth-Regulated Oncogene-beta (Groβ or CXCL2): macrophage inflammatory protein, secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells.
- Monocyte chemoattractant protein 1 (MCP-1): recruit monocytes, memory T cells, and dendritic cells to sites of inflammation produced by either tissue injury or infection. It’s mainly produced by monocytes, macrophages, and dendritic cells, which may induce adhesion molecule expression, tissue factor secretion, and smooth muscle cell proliferation in the context of inflammation. The adipose tissue is an important source for the release of MCP-1. It is the only adipocytokine able to impair insulin signaling and glucose uptake in skeletal muscle and its decrease facilitates insulin action in the muscle (Accattato et al., 2017).

Several studies have reported that physical activity can induce an acute-phase response characterized by an increase in multiple circulating cytokines and

chemokines, such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumor Necrosis Factor α (TNFα), Epidermal Growth Factor (EGF), and Monocyte Chemoattractant-1 (MCP-1); however, other studies have shown no significant changes in cytokine levels after exercise, probably due to differences in the physical activity protocols applied (Accattato et al., 2017). Also, it has been described how moderate exercise suppress Gro-alfa (Aqel et al., 2017) and, in experimental models, exercise down-regulated multiple inflammatory cytokines (Szalai et al., 2014), including Gro-alfa and Gro-beta, resulting in a systemic anti-inflammatory effect (Accattato et al., 2017).

4.2.3 Anti-inflammatory proteins: Angiogenin, Acrp30, Siglec 5

Other important immune factors present in mucosal secretions that change after prolonged exercise are anti-inflammatory proteins. Some of these proteins are:

- Angiogenin (Ang): associated with altered normality through angiogenesis (stimulates new blood vessels) and through activating gene expression that suppresses apoptosis (is associated with cancer and neurological disease). It has been reported that Ang interacts with endothelial and smooth muscle cells and induces a wide range of cellular responses like cell migration, invasion, proliferation, and formation of tubular structures (Gao and Xu, 2008; Tello-Montoliu et al., 2006).

- Adipokine (Acrp30 or adiponectin): is an adipocyte complement-related protein with roles in glucose and lipid homeostasis. It is a cell-signaling protein secreted by adipose tissue involved in regulating glucose levels as well as fatty acid breakdown (Berg et al., 2002). A negative correlation between obesity and circulating adiponectin has been well established, and adiponectin concentrations increase concomitantly with weight loss. Adiponectin decreases glucose production and lipid synthesis in the liver, promoting glucose and free fatty acid concentration decreases in blood. In addition, triglyceride production is decreased and fat oxidation and energy dissipation in the muscle are increased (Meier and Gressner, 2004).

Sialic acid-binding Ig-like lectin 5 (Siglec5): belongs to a novel subset of structurally related Ig superfamily of proteins that mediate protein-carbohydrate interactions,

specifically interacting with sialic acids in glycoproteins and glycolipids (Cornish et al., 1998).

4.3 Glycans

Among the various biological functions of carbohydrates, there are important roles that require interactions between cells and the surrounding matrix in order to assemble complex multicellular organisms. All cells have a series of independent or covalently linked sugars (monosaccharides) or sugar chains (oligosaccharides) called glycans. Because many glycans are found on the outer surface of cellular macromolecules, they can modulate or mediate a wide variety of events such as cell-cell, cell-matrix, and cell-molecule interactions that are critical to the development and function of an organism multicellular complex. They can also act as mediators in interactions between different organisms or linked to proteins, glycans can serve as regulatory switches. Glycans can, therefore, bind to diverse macromolecules forming glycoconjugates, such as glycoproteins and glycolipids (Figure 33) (Varki et al., 2009).

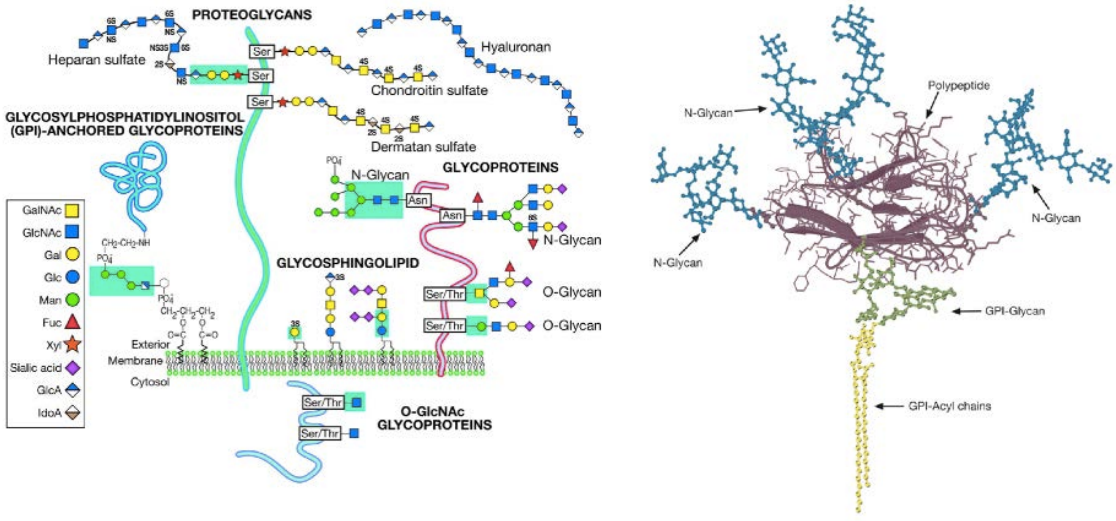


Figure 33. (a) Common classes of animal glycans; (b) schematic representation of a glycoprotein normally embedded in the membrane bilayer (Thy-1) with three N-glycans (blue), a glycosylphosphatidylinositol (GPI-glycan, green), a lipid anchor (yellow), and a polypeptide (purple). Retrieved from Essentials of Glycobiology (chapter 1), Varki et al. (2009).

Glycoproteins occur in all cellular compartments. The glycosylation generally involves the covalent attachment of glycans to proteins through serine, threonine, or asparagine residues, or to lipids like ceramide (which is comprised of sphingosine, a hydrocarbon amino alcohol and fatty acid). Complex glycans are mainly attached to secreted or cell surface proteins and do not cycle on and off of the polypeptide. Glycans play diverse roles within biological systems, especially in regard to inflammation and immune system activation (Figure 34) (Hart and Copeland, 2010). Almost all the key molecules involved in the innate and adaptive immune response are glycoproteins, which become key components of the effectors of the immune system. Also, the diversity of protein glycosylation plays an important role in the biosynthesis and biological activity of the glycoproteins involved in the recognition of antigens. Oligosaccharides are important in the synthesis, stability, recognition and regulation of the proteins themselves and in many of their various interactions (Rudd et al., 2001).

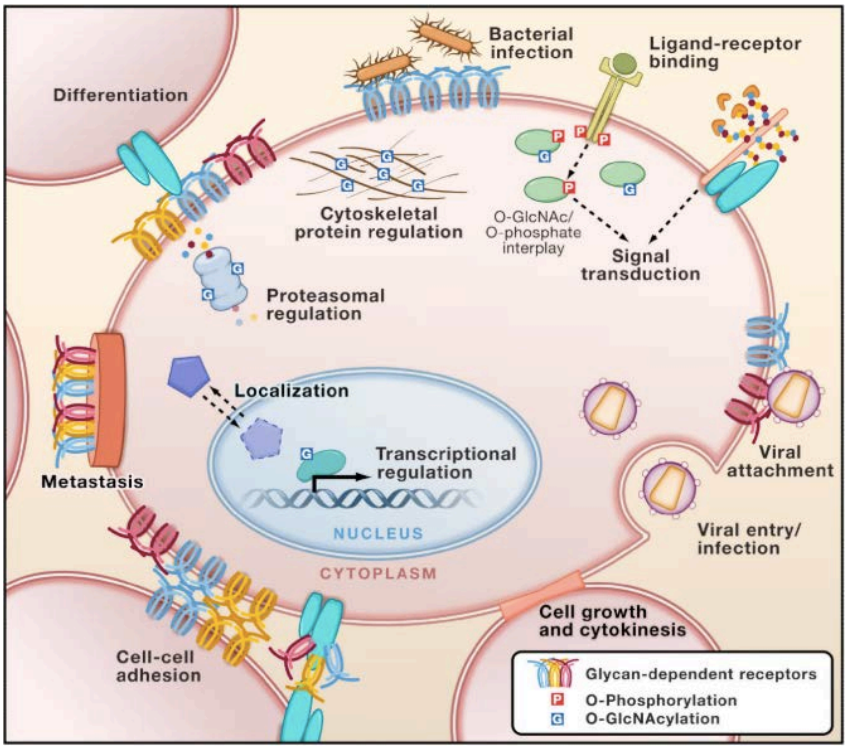


Figure 34. Glycans and cellular biology. Complex glycans play critical roles in intercellular and intracellular processes, which are fundamentally important to the development of multicellularity. Retrieved from Hart and Copeland (2010).

Inadequate nutritional practices can compound the negative effects of intensive exercise on immune function. Recent studies have demonstrated that ensuring adequate carbohydrate intake during prolonged strenuous exercise can minimize the effects of exercise on measures of innate and cell-mediated immunity (Bishop and Gleeson, 2009). Other trials validated that polysaccharide intake enhanced the immune system biomarkers in the blood of healthy adults (Ramberg et al., 2010), affected the immunomodulation through the glycosylation of proteins, and regulated leukocyte activity (Johnson et al., 2013; Rabinovich and Croci, 2012).

Other studies analyzing dietary plant polysaccharides and immunomodulatory potential, found increased macrophage cytotoxic and phagocytic activities, altered pro- and anti-inflammatory activities, Th1-Th2 balance, and altered expression of certain adhesion molecules. These polysaccharides could induce significant changes in the N-glycosylation status of serum glycoproteins. The increased sialylation observed can have a key role in the regulation of inflammatory processes (as in the case of IgG, where sialylation has been shown to function as a ‘switch’) (Alavi et al., 2011).

In this thesis, we wanted to investigate whether or not a glycan supplementation with a standardized dietary plant-derived polydisperse polysaccharide (Advance Ambrotose© complex powder, AA) resulted in a significant overall shift towards an increased sialylation of serum glycoproteins. AA is a saccharide supplement containing a standardized combination of plant polysaccharides (a source of mannose, galactose, fucose, xylose, glucose, n-acetyl-glucosamine, n-acetyl-neuraminic acid, and n-acetyl-galactosamine) that could regulate immunity through salivary glycol-modifications of proteins. These modifications could regulate the synthesis and/or degradation of pro- and anti-inflammatory molecules participating in the immune response of marathon runners.

5. Endurance exercise and nutrition

Being healthy is defined by the World Health Organization (WHO, https://www.who.int/) as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity", but lately some concepts have been redefined: "health is the ability to adapt and to self manage when individuals or communities face physical, mental, or social challenges" (Huber et al., 2011). Achieving and maintaining health is an ongoing process in everyone's hands, and it depends on what type of food we eat, how much water we drink, if we exercise, if we sleep well, what kind of work we do, and how much stress we accumulate over the years. Better nutrition means stronger immune systems, less illness, and better health. In addition, related to physical activity, optimal nutrition enhances performance and recovery from exercise (Thomas et al., 2016).

As it is well known, adequate energy intake is essential for the runner, especially with the intake of macronutrients and micronutrients, since it supports the optimal function of the body needed to perform. The majority of endurance practitioners are non-elite runners, and they generally focus on macronutrient intakes, disregarding the role of micronutrients that are mainly lost through sweat production. Therefore, an adequate diet should be designed considering several components like water with electrolytes, high vs low glycemic index carbohydrates, saturated vs unsaturated fat, animal vs plant-based protein, as well as the timing of nutrient intake (Lukaski, 2004).

Optimal nutrition and hydration can partially attenuate central or peripheral fatigue, thermal stress, dehydration, and/or endogenous glycogen store depletion (Costa et al., 2018). Nutritional intake influences the adaptation of training and recovery to achieve a good performance at the end with a proper body composition over time. Some effects on training adaptation due to a proper nutrition, are a set of physiological processes such as the size and number of mitochondria (Egan and Zierath, 2013), increase of oxidative enzymes expression, decrease of triglyceride reserves in adipose tissue, increase of intramuscular triglyceride reserves, or the increase of intramuscular fatty acid oxidation (Bartlett et al., 2014; Noakes, 2002).

With the strategic use of nutrition alone or in combination with training, adaptations can be achieved that improve the performance of the runner. This structured and planned process is called 'periodized nutrition' or nutritional training. One of the combined factors that determines the adaptive response to exercise training is the quality and quantity of nutrition in the pre- and post-exercise periods, aside from the duration, intensity, and the type of exercise (Jeukendrup, 2017). So, giving the deserved importance on what we eat as runners, both before, during and after physical activity, can make a substantial difference in performance and health.

5.1 Energy needs

As it is explained in section 2.1 (Figure 12), the energy in the body can be generated by three main ways: creatine phosphate metabolism, anaerobic glycolysis, and aerobic respiration. In the body we have different reserves from which to obtain energy with fat as the largest energy store in the body with around 9 kg compared to CHO with 600-700 g. The protein stores' size is more regulated, and the protein-breaking down by metabolic conversion to CHO is slow and well measured because these CHO are those that will feed certain organs such as kidneys, red blood cells, and brain. Regarding the weight of the whole-body composition, muscle is the largest organ contribution with 26 kg (or 37% from a 70-kg man), followed by fat tissue with 10.5 kg (15%), and bone with 10 kg (15%). Water represents up to 64% of total body weight or 83% of the weight of the body's water, fat and carbohydrate stores (Figure 35).

To analyze the energy a runner needs for each minute he runs, we need to know how much oxygen he needs. Knowing the oxygen consumption, it is possible to know how much energy can be produced (each liter of oxygen produces 20 kJ or 4.8 Kcal). For example, the oxygen cost of an elite runner at a pace of nearly 20 km/h at 67 mL O_2 per kg per min is around 4.7 liters, then he will be burning 93.8 kJ (22.42 Kcal) per minute. As 1 g of CHO provides 16.65 kJ (3.98 Kcal), the CHO stores can last 2h05min compared with the fat stores that provide 37.5 kJ (8.96 Kcal) per gram and then can fuel exercise for 59h01min (much more than with CHO). But the intensity plays an important role in the CHO or fat fuel uses. When the exercise intensity exceeds about 80% of VO_{2max}, all the energy comes from CHO metabolism

and lasts little. Training and experience are factors that a runner has to not exceed from around 67% of VO_{2max} and then use both fat and CHO for energy, being more successful performance when one is capable to extract most of the energy from fat (Noakes, 2002).

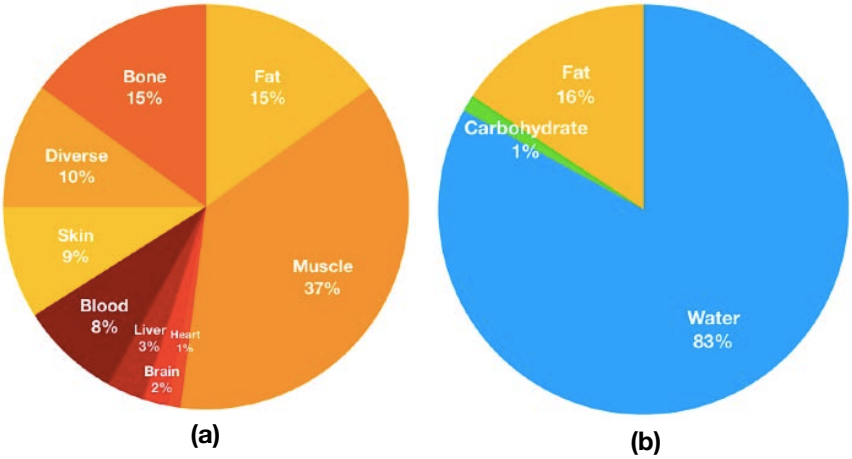


Figure 35. Percentage by weight of the different organs of the body (a) and of the body water, fat and CHO stores (b). Adapted from Noakes (2002).

The level of training and the diet (including fasting), affect the amount of CHO that can be stored (the muscle and liver have the capacity to store glycogen). Also, trained runners have about 10% less body weight as fat and have an improved utilization and availability of nutrients (Noakes, 2002). In addition, a good diet can accelerate recovery, because a rapid restoration of body glycogen reserves accelerates the repair of muscle damage and the recovery of immune function (Thomas et al., 2016).

5.2 Macronutrients

Macronutrients are a class of chemical compounds which humans consume in the largest quantities. The main primary macronutrients are carbohydrate, protein, lipids, water, and fiber (Table 3). Most of them provide the bulk of energy necessary, while others such as fiber and water do not provide energy (Prentice, 2005). Several sports medicine and nutrition-related organizations recommend specific amounts of energy, macronutrients, and micronutrients for sustained physical activity events in

order to maintain body weight, replenish glycogen stores, and provide sufficient proteins to build and repair working tissues (Thomas et al., 2016). Macronutrient intake by itself doesn't predict an ultra-endurance performance (predicting finishing times), but habitual dietary fat for example does (Mahoney et al., 2016). What we eat influences our body´s capabilities to adapt to the training, performance and to post-exercise recovery (Williamson, 2016).

Intensity, duration, and food intake will largely determine how much fuel is being sourced from carbohydrates (CHO), protein, and fat (as explained in 5.1). Although all three are being used as sources of energy at any given time, the intensity and duration of exercise are primary factors that determine the extent to which one is used over another.

Table 3. List of the main macronutrients.

Carbohydrates	Protein	Fats				Water	Fiber
	Standard amino acids:	Saturated fats	Monounsaturated fats	Polyunsaturated fats	Essential fatty acids (EFA)		
Glucose	Alanine	Butyric acid (C4)	Myristol	Linoleic acid (LA) -EFA	Omega-3 fatty acid		
Sucrose	Arginine	Caproic acid (C6)	Pentadecenoic	α-Linolenic acid (ALA) - EFA	Omega-6 fatty acid		
Ribose	Aspartic acid (aspartate)	Caprylic acid (C8)	Palmitoyl	Stearidonic acid (SDA)			
Amylose	Asparagine	Capric acid (C10)	Heptadecenoic	Arachidonic acid (ETA)			
Amylopectin	Cysteine	Lauric acid (C12)	Oleic acid	Timnodonic acid (EPA)			
Maltose	Glutamic acid (glutamate)	Myristic acid (C14)	Eicosen	Clupanodonic acid (DPA)			
Galactose	Glutamine	Pentadecanoic acid (C15)	Erucic acid	Cervonic acid (DHA)			
Fructose	Glycine	Palmitic acid (C16)	Nervonic acid				
Lactose	Histidine	Margaric acid (C17)					
	Isoleucine (BCAA)	Stearic acid (C18)					
	Leucine (BCAA)	Arachidic acid (C20)					
	Lysine	Behenic acid (C22)					
	Methionine	Lignoceric acid (C24)					
	Phenylalanine	Cerotic acid (C26)					
	Proline						
	Serine						
	Threonine						
	Tryptophan						
	Tyrosine						
	Valine (BCAA)						

5.2.1 Carbohydrates

Glucose is the primary energy source, the only energy substrate in the body that works exclusively to provide energy to cells. During exercise, glucose levels in blood depend on exercise intensity, energy status, food intake, and glycogen storage levels (when there is reduced availability there is fatigue) (Lee et al., 2017). One way to replenish glycogen, maintain blood glucose levels, and enhance performance (especially for high-intensity activity) is the carbohydrate consumption before or during prolonged exercise (Vandenbogaerde and Hopkins, 2011). For some time now, it has been demonstrated that daily dietary CHO (3-12 g/kg/day) and CHO intake during endurance events (30-110 g/h) can enhance performance at individually tolerable intake rates (Costa et al., 2018). Carbohydrate intake recommendations depend on the duration of exercise (Figure 36) and also the CHO type to ingest can improve the performance delaying the fatigue. For example, there's an increased oxidation rate of these exogenous CHO with 2 glucose:1 fructose than with glucose alone because different transporters are used (Jeukendrup, 2014).

Before a competition, it is necessary to take into account an overcompensation of glycogen and a pre-race meal 4 h before improves performance up to 2-3% (Hawley et al., 1997). There are studies, therefore, that say that with 24-36 hours of rest before a race and an intake of 8-12 g/kg/day, you can also fill the stores (Bussau et al., 2002). After the race, recovery from exhaustive muscle glycogen depletion is not a simple matter and often requires days, rather than hours to recover the phosphagen and lactic acid metabolic systems. Consuming carbohydrates immediately post-exercise to coincide with the initial rapid phase of glycogen synthesis has been used as a strategy to maximize rates of muscle glycogen synthesis, especially if there is another bout of exercise within 8 h after (otherwise normal meals will replace glycogen stores) (Beck et al., 2015).

Many recreational runners don't have access to professional nutrition advice, rather they utilize anecdotal evidence and rely on popular media to deliver nutrition information and recommendations. For example, in the past several years, some winners of major ultramarathon races have reported the use of low-carbohydrate diets, high-fat diets (LCHF), keto diets,… and thus, interest in these diets is increasing among recreational ultra-endurance athletes (Mahoney et al., 2016).

When exercise duration is long enough (more than 10 h) and exercise intensity low enough (for example 45–60% of VO_{2max}, section 2.1.1), it is bio energetically plausible that ketogenic adaptation (the body uses primarily fat for energy) may enhance ultra-endurance performance (Costa et al., 2018). It seems that these diets facilitate the use of fat by the muscle, but do not allow the muscle to adapt quickly to changing substrate, for example, glucose. This makes the athletes less efficient in the sprints or changes of rhythm, common in competition. However, more research must be done.

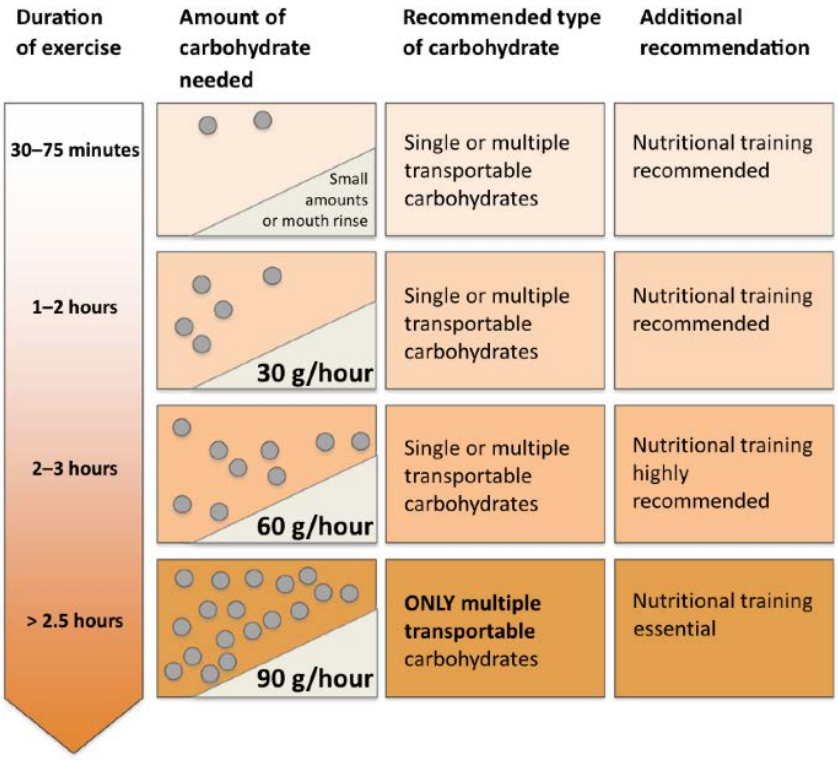


Figure 36. Carbohydrate intake guidelines. CHO intake recommendations increase with increasing duration of exercise and also the CHO type and nutritional training vary. Retrieved from Jeukendrup (2014).

5.2.2 Fats

Fat is of particular interest among macronutrients for endurance athletes, as it provides energy, fat-soluble vitamins, and essential fatty acids, which are associated with a lower risk of cardiovascular disease and mortality (Dehghan et al., 2017). Fats are used as a primary energy source when carbohydrate availability is low or when the intensity in endurance events is low/medium; in fact, endurance training

improves an athlete's ability to oxidize fat during exercise. Fat, in the form of free fatty acids in plasma (associated with albumin), intramuscular triglycerides, and adipose tissue, provides a relatively abundant fuel substrate for muscle, especially when endurance training is done (Thomas et al., 2016). In particular, medium-chain fatty acids are preferred for oxidation, since they enter circulation faster and are primarily absorbed by the liver. The use of fat during exercise improves cardiovascular health profiles because it reduces the resting levels of total cholesterol and triglycerides. Some types of fat in addition to providing energy, also play an important role in recovery (Lee at al., 2017).

Exercise intensity affects fatty acid oxidation (FAox), which is optimal between 45 and 65% VO_{2max} (maximal fat oxidation). At higher intensities FAox is reduced due to limitations within fatty acid transport across the cell and mitochondrial membranes. The point at which FAox reaches a maximum and begins to decline is referred to as the crossover point (Figure 37). When the exercise intensities exceed the crossover point (~65% VO_{2max}), the body utilizes CHO as the predominant fuel source for energy supply (Purdom et al., 2018).

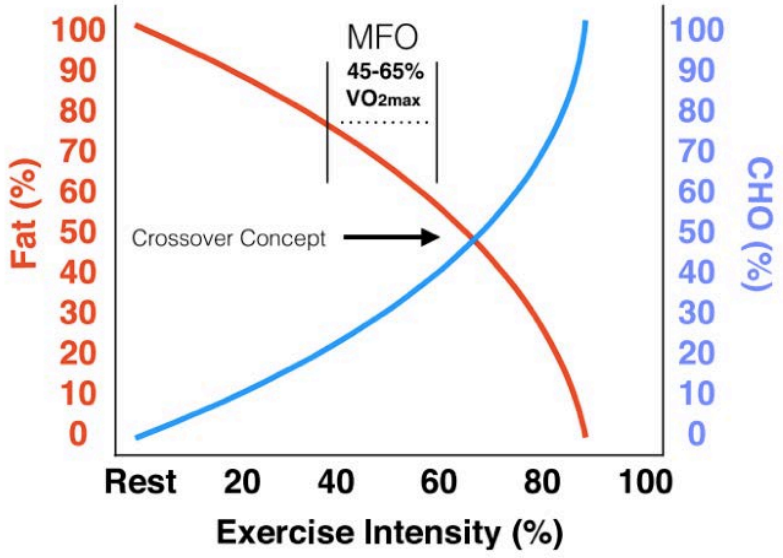


Figure 37. The crossover concept: when exercise intensity increases, there is a decrease in energy derived from fat and an increase in CHO. The crossover point describes when the CHO contribution to substrate oxidation supersedes that of fat. MFO: maximal fat oxidation. Retrieved from Purdom et al. (2018).

Long-chain omega-3 polyunsaturated fatty acids for example, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), reduce inflammation, muscle soreness, perception of pain from exercise (Lee et al., 2017), and their blood levels are inversely related to risk for all-cause mortality and fatal cardiovascular disease events (Jackson and Harris, 2018).

When a high-carbohydrate diet is consumed, the intake of fats is similar to that recommended for the general population of 20 to 35%. In this way, it is possible to maintain performance and health, but it is recommended that the consumption of saturated fats does not exceed 10% and include sources of essential fatty acids (Williamson, 2016).

There is another metabolic state in which some of the body's energy supply comes from ketone bodies in the blood, in contrast to a glycolysis state in which blood glucose provides energy. This ketosis state occurs when the body is metabolizing fat at a high rate and converting fatty acids into ketones. It has been seen how this state improves physical endurance by altering fuel competition for oxidative respiration, decreases muscle glycolysis and plasma lactate concentrations while providing an alternative substrate for oxidative phosphorylation. Ketosis also increases intramuscular triacylglycerol oxidation during exercise, even in the presence of normal muscle glycogen, co-ingested carbohydrate and elevated insulin (Cox et al., 2016).

In consequence, fat oxidation rates can be improved with dietary strategies such as fasting, acute fat intake before exercise, and chronic exposure to diets high in fat and low in carbohydrates (low-carbohydrate high-fat diets, LCHF). Although there has been a recently revived interest in chronic adaptation to LCHF diets, there are divergences in their effect. There is evidence to suggest that improved rates of fat oxidation can only match the exercise performance achieved with diets or strategies that promote a high availability of CHO at moderate levels or intensities (Thomas et al., 2016).

On the other hand, LCHF diets consistently improve markers of cardiovascular risk, lowering elevated blood glucose, insulin, triglyceride, ApoB, and saturated fat (especially palmitoleic acid) concentrations, reducing small dense LDL particle numbers, glycated hemoglobin levels, blood pressure, and body weight, while

increasing low HDL-cholesterol concentrations and reversing non-alcoholic fatty liver disease (Noakes and Windt, 2017). This LCHF provides athletes with several ketosis-induced adaptations that reduce oxidative stress and might influence rates of recovery from demanding exercise. Some highly adapted runners consuming less than 10% of energy from carbohydrates are able to oxidize fat at greater than 1.5 g/min during progressive intensity exercise and consistently sustain rates of fat oxidation exceeding 1.2 g/min during exercise at ~65% VO_{2max}. The remaining energy that is needed is derived from the oxidation of blood lactate, ketone bodies and glucose derived from gluconeogenesis, without them needing to ingest exogenous fuels, specifically carbohydrate during a long endurance race (Noakes et al., 2014).

5.2.3 Proteins

Proteins serve as building blocks of hormones and enzymes used in all cells and tissues of the body, such as muscles. Protein is a critical nutrient that requires considerable attention on the part of the athlete to ensure adequate recovery of exercise and to promote optimal adaptation between training sessions (Williamson, 2016). Marathoners, for example, when participating in endurance training and races, have higher needs than those required for the general population due to the need to repair damaged muscles and synthesize new muscle proteins. In general, athlete protein intakes of 1.3 to 2.0 g/kg/day are recommended (unlike 0.8 g/kg/day recommended by WHO to sedentary people), because they need more synthesis of muscle proteins, facilitate training adaptations and prevent the loss of lean muscle mass (Lee et al., 2017). In addition, the advisable thing is not to use the body weight to establish the amount of proteins that each person needs, but the one in lean weight (body weight-body fat = fat-free mass or lean weight in kg). The requirements may vary depending on the trained person (experienced athletes require less), the training (sessions with higher frequency and intensity require more protein), the availability of CHO and, above all, the availability of energy (Thomas et al., 2016).

Endurance exercise implies maintenance of skeletal muscle integrity specifically important in the early post-exercise recovery period. This integrity can be associated, for example, with an increase in myofibrillar and/or mitochondrial protein, with synthesis and breakdown that depends on the protein's half-life, and is controlled by

several intracellular systems. These systems require intracellular energy coming specifically from macronutrient intake like proteins (Smiles et al., 2016).

It has been shown that body protein reserves provide up to 10% of the total energy used during endurance exercises and their contribution is influenced by many factors such as the intensity, duration or level of glycogen available in the body. Due to running, a loss of muscle mass occurs, taking place in all muscle groups, specifically in the lower region of the leg or calf. This loss is mainly due to eccentric contractions. This endogenous protein lost can be reduced by ensuring adequate reserves of glycogen before the activity and by consuming adequate energy during the prolonged activity, while promoting post-activity recovery. The rate of muscle degradation is accelerated when the oxidation of muscle proteins exceeds the synthesis, this occurs in intense and long-lasting exercises such as a marathon. Also, the immediacy of protein intake in the diet after exercise is essential for optimal recovery, suggesting a minimum of 20 g of protein within 30 to 60 minutes after exercise to optimally stimulate muscle protein synthesis and mitigate any existing decomposition that is occurring as a result of prolonged exercise (Williamson, 2016).

5.3 Micronutrients

Macronutrients provide sources of energy needed to fuel the body, maintain cellular hydration, and provide the body structure to perform work. Micronutrients enable the use of macronutrients for all physiologic processes. In Table 4 the main micronutrients are listed and they differ from macronutrients in their key characteristics, being regulators of health, function, and work performance. Water, proteins, carbohydrates, and fat are consumed in large amounts (around 100 grams per day), whereas vitamins and minerals are ingested in much smaller amounts (milligrams to micrograms per day), reflecting the turnover rates in the body and specific functions (Lukaski, 2004).

Many of the metabolic pathways involved in running require micronutrients. Endurance training increases the demand for micronutrients because there is a biochemical muscle adaptation that needs them. When a runner restricts energy consumption, wants to lose weight or his diet is unbalanced, he will surely be

consuming sub-optimal amounts of micronutrients and will have to supplement them (most crucially with calcium, vitamin D, iron and some antioxidants) (Thomas et al., 2016).

Table 4. List of the main micronutrients.

Vitamins	Trace Minerals	Antioxidants
Vitamin B complex	Boron	
Vitamin B1 (thiamin)	Cobalt (as a component of vitamin B12)	
Vitamin B2 (riboflavin)	Chromium	
Vitamin B3 (niacin)	Copper	
Vitamin B5 (pantothenic acid)	Iodine	
Vitamin B6 group (Pyridoxine, Pyridoxal-5-Phosphate, Pyridoxamine)	Iron	
Vitamin B7 (biotin)	Manganese	
Vitamin B9 (folate)		
Vitamin B12 (cobalamin)		
Choline		
Vitamin A (e.g. retinol)		
Vitamin C (Ascorbic acid)		
Vitamin D		
Ergocalciferol		
Cholecalciferol		
Vitamin E (tocopherols and tocotrienols)		
Vitamin K		
Vitamin K2 (menaquinone)		
Carotenoids		
Alpha carotene		
Beta carotene		
Cryptoxanthin		
Lutein		
Lycopene		
Zeaxanthin		
Vitamin K1 (phylloquinone)		

The biological processes directly related to performance are supported by a variety of vitamins and minerals. Low levels of calcium, iron, B vitamins, and vitamin D for example, are associated with an increased risk of injuries such as stress fractures in

the lower extremities. Some of the micronutrients that can most affect a runner are (Lee et al., 2017):

- Vitamin D: its levels correlate with aerobic performance.
- B complex vitamins (thiamin, riboflavin, niacin, pyridoxine, folate, biotin, pantothenic acid and choline): regulate energy metabolism and therefore sports performance because they modulate the synthesis and degradation of carbohydrates, fats, proteins, and bioactive compounds.
- Vitamin E: plays an important secondary role in the recovery process, being an antioxidant in cell membranes and in subcellular structures.
- Beta-carotene (precursor of vitamin A): acts as an antioxidant to reduce muscle damage and improve recovery after exercise.
- Magnesium: important for energy metabolism, as well as for nerve and muscle function. Its deficiencies can lead to muscle weakness, muscle spasms, and impaired CK and lactate response to exercise.
- Iron: directly affects the physical performance and its impairment in muscle function, limiting work capacity.
- Zinc: required for a variety of functions including protein synthesis, cell function, glucose use, hormonal metabolism, immunity, and wound healing.
- Chromium: is a provisionally essential mineral that intervenes in the regulation of glucose, lipid, and protein metabolism by enhancing the action of insulin at the cellular level. In athletes, there may be a greater need to consume it because they excrete it more than sedentary.

5.4 Hydration

Water is the most essential nutrient of the body and plays a critical role in thermoregulatory and overall function. Water is undergoing continuous recycling, functioning as a solvent and regulating cell volume. Water balance is controlled by thirst and renal function (with vasopressin, the antidiuretic hormone) (Lee et al., 2017). Muscular work generates heat that can be aggravated by environmental

conditions, but sweat allows its dissipation maintaining the body temperature within the acceptable ranges. Being appropriately hydrated contributes to optimal health and exercise performance. In addition to the usual daily water losses from respiration, gastrointestinal, renal, and sweat sources, athletes need to replace sweat losses more frequently that sedentary. Sweat contains substantial but variable amounts of electrolytes like sodium, with lesser amounts of potassium, calcium, and magnesium. Runners should strive to undertake fluid management strategies before, during and after exercise that maintain hydration and thus preserve homeostasis, optimal body function, performance and perception of well-being (Thomas et al., 2016).

The strenuous physical exertion that is a marathon with the possibility to suffer heat stress, results in an increase in blood flow to the skeletal muscles and skin, consequently renal blood flow decrease to 25% of levels at rest. This reduction in blood supply leads to ischemic tubular damage (Mansour et al., 2017). Consequently, severe dehydration after a marathon can be a precursor to acute kidney injury (AKI). One of the blood biomarkers that can detect AKI is creatinine. This protein is a breakdown product of creatine phosphate in muscle (Figure 12a), and is usually produced at a fairly constant rate by the body and it is muscle mass depending. Creatinine can be elevated to some degree because of skeletal muscle size and activity, but it has been a direct indicator together with glomerular filtration rate (GFR), of a transient reduction in renal filtration function (McCullough et al., 2011). Mansour et al. (2017) found significant increases in serum creatinine after the marathon and more than 80% of the runners developed at least stage 1 AKI (defined as a 1.5- to 2-fold increase or 0.3-mg/dL increase in serum creatinine level from pre-race value).

The daily contribution of electrolytes is very important for a runner, because they are capable of conducting an electrical current in solution and this property is critical in transmitting nerve impulses and prompting muscle contraction (skeletal and cardiac). Sodium (Na^+), calcium, and potassium (K^+) must be closely regulated, because a failure of potassium regulation, for example, can have serious consequences on nerve conduction, skeletal muscle function, and most significantly, on cardiac muscle contraction and rhythm (Figure 38) (Betts et al., 2017). The Na^+-K^+ pump (located in the sarcolemma and a large fraction in the t-tubular membranes, see Figure 11b) is a

regulator of the transport and distribution of Na^+ and K^+ across cell membranes with the main role in the excitability and contractile performance in skeletal muscle. Training upregulates and inactivity downregulates the content of Na^+-K^+ pumps in skeletal muscle (Clausen, 2003).

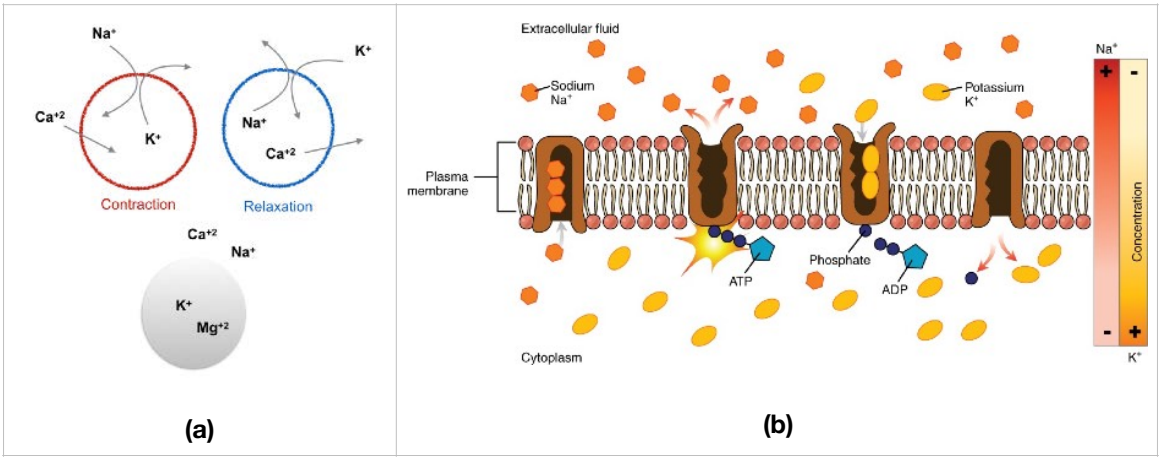


Figure 38. Diagram of intra and extracellular electrolyte distribution (a). The sodium-potassium pump powered by ATP to transfer sodium out of the cytoplasm and potassium into the cell (b). Retrieved from Betts et al. and OpenStax, Anatomy and Physiology: Fluid, Electrolyte, and Acid-Base Balance (2017).

As little as a 2% reduction in body mass due to dehydration (refers to the process of losing body water and leads to hypohydration) has been said to result in performance decrements as well as hemorheology, metabolic dysregulation, cognitive function, mood, heat intolerance, and cardiovascular strain (Williamson, 2016). To evaluate the degree of dehydration with both cutaneous (sweating) or renal (urination) water losses, acute decrease in body weight has been used as the gold standard, because it reflects mainly a decrease in total body water and not energy substrates like fat or protein (Lee et al., 2017). Also the urine color, due to its concentration, is an indicator of hydration status, this concentration rises substantially throughout the race and increasingly becomes less reliable with duration (Williamson, 2016).

Other studies defend weight changes before and after an endurance exercise do not provide an accurate indication of hydration status specially for endurance athletes, because the change can be attributed to substantial breakdown of body tissues such as adipose and muscle and increases in weight can result from reduced diuresis (by

activation of vasopressin secretion and the angiotensin–renin–aldosterone mechanism), as well as decreases in intracellular osmolytes including glycogen, proteins, and triglycerides. And also weight loss greater than 2% does not necessarily have serious adverse consequences on performance (Williamson, 2016). There are findings in which athletes regularly lose 5–6% of their body mass during ultra-endurance exercise lasting 5 to 24 h, whilst maintaining proper fluid allostasis and without developing any medical complications. In addition, it has been assessed that the best performing athletes are usually the most ‘dehydrated’, with some body mass losses of >6% (Tam and Noakes, 2013).

Prolonged strenuous endurance exercise can alter physiological processes associated with the fluid imbalance and electrolyte levels that with proper nutrition can help mitigate the majority of the associated problems (Manore et al., 2000). But hydration during endurance events is not easy and hyperhydration has become increasingly common, being the most reported medical complication in ultradistance races. Hyperhydration can lead to life-threatening hyponatremia by altering the proportion of sodium in the blood and provoke brain swelling due to the entrance of excess water transported through osmosis. In order to prevent this, current research suggests that the most appropriate strategy to maintain hydration and avoid hyponatremia associated with exercise (EAH), during and immediately following exercise, is "drink up thirst" (Williamson, 2016).

EAH can occur during or up to 24 h after a prolonged exercise with large volumes of water consumed. Symptoms usually appear when serum sodium levels are below 135 mmol/L regardless of the presence or absence of signs and symptoms. The normal sodium range either during or outside of exercise is 135-145 mmol/L. Most of the clinical manifestations are related to headaches, nausea and vomiting, in the early phase, and with disorientation, confusion, coma, seizures, and signs of pulmonary edema, in the later phase. Also, EAH may lead to rhabdomyolysis (see section 2.2.1) through changes in the skeletal muscle membrane by osmotic shock and activation of reactive oxygen species (ROS) along with increases in intracellular calcium that activate intracellular proteases and lead to a cellular breakdown. There is a relationship between rhabdomyolysis and hyponatremia because rhabdomyolysis can lower sodium and low sodium can induce rhabdomyolysis (Hoffman et al., 2015). Rhabdomyolysis leads to local and systemic inflammation

and release of interleukin-6 (IL-6), which may increase arginine vasopressin (AVP) levels that further reduce serum sodium. Anti-inflammatories (NSAIDs) use may also contribute to excess AVP release (Figure 39) (Hew-Butler et al., 2017). Also, NSAIDs consumption before or during endurance races could contribute to the alteration of renal function aggravated by dehydration, and lead to the race abandon (more than 60.5% who finish and 46.4% who do not finish an ultradistance race have consumed NSAIDs) (Hoffman and Fogard, 2011).

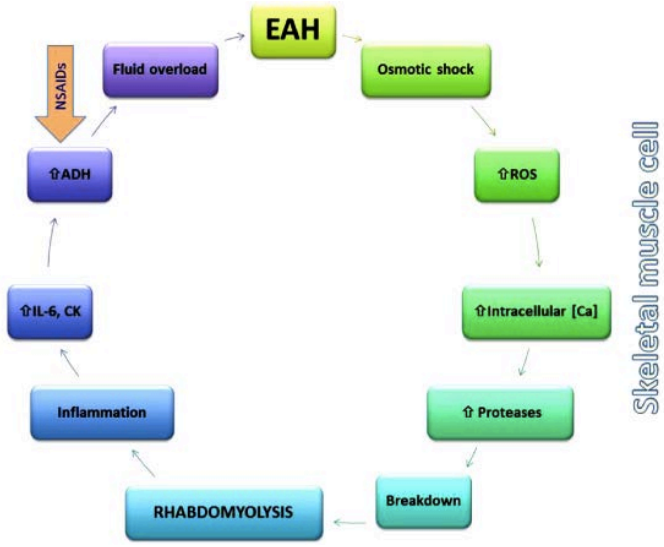


Figure 39. Possible bidirectional relationship between exercise-associated hyponatremia (EAH) and rhabdomyolysis. Retrieved from Hew-Butler et al. (2017).

5.5 Nutrition and hydration status biomarkers

Athletic performance and recovery from exercise are enhanced by optimal nutrition as it is explained in this chapter. When nutritional intake is inadequate or there is disordered eating in athletes, functional performance is impaired. Individual nutritional needs depend largely on exercise specific bioenergetic demands as well as on an athlete's metabolic tolerance, needs, and preferences. A way to identify individual deficiencies and track changes, is the frequent monitoring of macronutrient and micronutrient intake, especially as runners' training volume and nutritional demands increase. Some examples of options for well-studied biomarkers in each category are (Lee et al., 2017):

Macronutrient metabolism: glucose, triglycerides, free fatty acids, cholesterol, lipids, total protein, albumin, globulin, blood urea nitrogen, amino acid, etc.

Micronutrient metabolism: vitamin D, B vitamins, vitamin E, magnesium, iron, zinc, chromium, etc.

Hydratation status: body mass, plasma/serum osmolality, plasma sodium, blood creatinine, arginine vasopressin, urine osmolality, urine color, salivary and tear osmolality, thirst, etc. Because sweat is hypotonic, exercise-induced dehydration leads mainly to a decrease of extracellular fluid volume (hypertonic hypovolemia due to water loss from the plasma). Blood biomarkers of hemoconcentration have thus been widely used as indexes of dehydration also (Tam and Noakes, 2013).

Being able to find in the non-elite runners a relationship between blood biomarkers, nutrient intake and performance in a marathon can serve to develop the best and healthiest nutritional strategy, specifically when there are so many possibilities on the table.

5.6 Whole-body bioimpedance measurements

The BIA measurements phase-sensitive at 50 kHz in the BIVA model are extensively used in the evaluation of hydration status (Lukaski and Piccoli, 2012). After an intervention like an endurance running such as a marathon, the change in hydration with a gain or loss of fluids can be evaluated by BIVA (Lukaski et al., 2019). This method detects little changes in the fluid distribution before and after the marathon.

The evaluation of the state of hydration, nutrition and training procedures, among others, can give very valuable information to be able to establish the homeostasis of the runner without incurring unwanted adverse effects.

6. Hypothesis

Strenuous exercise such as a marathon in amateur or non-elite runners can cause changes in biomarkers associated with the pathology of several tissues such as skeletal and cardiac muscle, as well as impairing renal function and immunity. Concentration and activity of creatine kinase, C-reactive protein, creatinine, urea, sodium, mineral and lipid profile, cardiac high-sensitive troponin T, amino-terminal pro-B type natriuretic peptide and ST2 in blood, or IgA, antimicrobial proteins, anti-inflammatory proteins and chemokines in saliva, are all altered in response to a bout of prolonged exercise. Such changes are transient and in most cases, full recovery occurs within 48 h, without any apparent long-term adverse consequences. But a sum of acute effects can become chronic, especially when you do not run a single marathon a year.

We hypothesized that non-elite marathon runners, who experienced changes in these biomarkers would be more prone to tissue damage, injury, inflammation, cardiac, and renal impairment, infection and depressed performance, especially caused by inappropriate body weight and a poor training plan, recovery time, and diet.

7. Objectives

Main objective

The main objective of this doctoral thesis is to analyze the impact of running in non-elite runners over different metabolic systems, and how this is affected by previous training hours, race time, recovery time, body weight, diet, and specific supplementation.

Specific objectives

Through a diet, health and anthropometry questionnaire, an analysis of bioimpedance vectors and saliva, blood and urine samples were taken at different time intervals (pre-race, post-race and 48 h post-race), evaluate the changes related to:

1. Muscle damage and inflammation: by quantifying creatine kinase (CK), and C-reactive protein (CRP), and analyzing the bioelectrical impedance vector (BIVA).

2. Cardiovascular health: by quantifying high-sensitivity cardiac troponin T (Hs-TnT), amino-terminal pro-B type natriuretic peptide (NT-proBNP), suppression of tumorigenicity 2 (ST2), and lipid profile (triglycerides, low density lipoprotein, high density lipoprotein and cholesterol).

3. Immunity and infection: by quantifying salivary immunoglobulin A (sIgA), anti-inflammatory proteins (angiogenin, adipokine, sialic acid-binding Ig-like lectin 5), pro-inflammatory chemokines (growth-regulated oncogene-alpha, growth-regulated oncogene-beta, monocyte chemoattractant protein 1), and antimicrobial proteins (lactoferrin, lysozyme).

4. Renal health: by quantifying creatinine, urea, and sodium, and analyzing the bioelectrical impedance vector (BIVA).

Chapter 2: The Dynamics of Cardiovascular Biomarkers in non-Elite Marathon Runners

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Title: The Dynamics of Cardiovascular Biomarkers in non-Elite Marathon Runners

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CORRESPONDENCE

The Dynamics of Cardiovascular Biomarkers in non-Elite Marathon Runners

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Abstract The number of recreational/non-elite athletes participating in marathons is increasing, but data regarding impact of endurance exercise on cardiovascular health are conflicting. This study evaluated 79 recreational athletes of the 2016 Barcelona Marathon (72% men; mean age 39 ± 6 years; 71% ≥35 years). Blood samples were collected at baseline (24–48 h before the race), immediately after the race (1–2 h after the race), and 48-h post-race. Amino-terminal pro-B type natriuretic peptide (NT-proBNP, a marker of myocardial strain), ST2 (a marker of extracellular matrix remodeling and fibrosis, inflammation, and myocardial strain), and high-sensitivity troponin T (hs-TnT, a marker of myocyte stress/injury) were assayed. The median (interquartile range, IQR) years of training was 7 (5–11) years and median (IQR) weekly training hours was 6 (5–8) h/week, respectively. The median (IQR) race time (h:min:s) was 3:32:44 (3:18:50–3:51:46). Echocardiographic indices were within normal ranges. Immediately after the race, blood concentration of the three cardiac biomarkers increased significantly, with 1.3-, 1.6-, and 16-fold increases in NT-proBNP, ST2, and hs-TnT, respectively. We found an inverse relationship between weekly training hours and increased ST2 ($p = 0.007$), and a direct relationship between race time and increased hs-TnT ($p < 0.001$) and ST2 ($p = 0.05$). Our findings indicate that preparation for and participation in marathon running may affect multiple pathways affecting the cardiovascular system. More data and long-term follow-up studies in non-elite and elite athletes are needed.

Keywords Biomarkers · Marathon · ST2 · Hs-TnT · NT-proBNP · Sports

Associate Editor Craig Stolen oversaw the review of this article

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The number of recreational/non-elite athletes participating in marathons is increasing, but data regarding impact of endurance exercise on cardiovascular health are conflicting [1]. Strenuous exercise may increase the circulating concentrations of cardiac biomarkers that are used to monitor heart health and disease. Here, we examined dynamics of a panel of cardiac biomarkers in non-elite athletes completing the Barcelona Marathon in 2016.

The local ethics committee approved the study, and all participants provided written informed consent. The study sample included 79 recreational athletes (72% men; mean age 39 ± 6 years; 71% ≥ 35 years) who responded to a call for volunteers (http://www.emmaroca.com/es/forms/estudi-cientific-perfil-de-lultrafondista/). Blood samples were collected at baseline (24–48 h before the race), immediately after the race (1–2 h after the race), and 48-h post-race. Biomarkers were measured using commercially available assays for amino-terminal pro-B type natriuretic peptide (NT-proBNP, a marker of myocardial strain), ST2 (a marker of extracellular matrix remodeling and fibrosis, inflammation, and myocardial strain) [2], and high-sensitivity troponin T (hs-TnT, a marker of myocyte stress/injury).

The median (interquartile range, IQR) years of training was 7 (5–11) years and median (IQR) weekly training hours was 6 (5–8) h/week, respectively. The median (IQR) race time (hours:min:sec) was 3:32:44 (3:18:50–3:51:46). Echocardiographic indices measured after the race were within normal ranges (left ventricular (LV) ejection fraction 62 ± 5%; LV end diastolic diameter 50 ± 5 mm; indexed LV mass 94 ± 20 g/body surface area; tricuspid annular plane systolic excursion 26 ± 3 mm). Although not the focus of the present report, we also measured

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Table 1 Cardiovascular biomarker dynamics in recreational marathon runners

Biomarker	Baseline	Immediately post-race	48-h post-race	*p* value*	*p* value**
NT-proBNP, ng/L	70 (70–70)	92 (70–147)	70 (70–70)	<0.001	0.29
ST2, ng/mL	34.2 (24.7–40.9)	54.2 (38.2–72.4)	33.7 (28.9–42.3)	<0.001	0.53
hs-TnT, ng/L	2.9 (1.7–7)	46.9 (24.1–91.1)	4.7 (2.4–8.85)	<0.001	<0.001
≥Cut-off point					
NT-proBNP, ≥125 ng/L (%)	0	30.7	1.4	<0.001	1.00
ST2, ≥35 ng/mL (%)	48.7	86.7	48.6	<0.001	1.00
hs-TnT, ≥14 ng/L (%)	10.4	88.3	17.9	<0.001	0.03

Values are reported as median (IQR); * 1–2-h post-race vs. baseline; ** 48-h post-race vs. baseline. Wilcoxon paired data test was used for continuous variables; McNemar test was used for categorical variables

creatinine phosphokinase (CK) as a marker of skeletal muscle breakdown. CK increased from a baseline level of 165 U/L (125–239 U/L) to 518 U/L (377–709 U/L) immediately post-race and 692 U/L (462–1032) at 48 h (both $p < 0.001$ relative to pre-race values). CK dynamics were not associated with age, years of training, weekly hours of training, or performance of the athletes.

Table 1 shows biomarker values. A significant number of runners had ST2 (48.7%) and hs-TnT (10.4%) concentrations above accepted cutoff point for individuals without cardiac disease at baseline and 48-h post-race. Baseline hs-TnT correlated directly with weekly training hours ($p = 0.01$) and correlated inversely with race completion time ($p = 0.009$). None of the biomarkers at baseline correlated with years of training or weekly training hours.

Immediately after the race, blood concentration of the three cardiac biomarkers increased significantly, with 1.3-, 1.6-, and 16-fold increases in NT-proBNP, ST2, and hs-TnT, respectively (Table 1; all $p < 0.001$). NT-proBNP and ST2 concentrations returned to baseline 48 h after the race, while hs-TnT concentrations remained 60% higher than baseline levels (Table 1, $p < 0.001$). Age and years of training showed no significant relationships with the dynamics of the biomarkers. However, we found an inverse relationship between weekly training hours and increased ST2 ($p = 0.007$), and a direct relationship between race time and increased hs-TnT ($p < 0.001$) and ST2 ($p = 0.05$). In multivariable linear regression analyses that included age, sex, and variables with a p value ≤0.10 in correlation analyses, race time remained independently associated with elevations in ST2 ($p = 0.03$) and hs-TnT ($p < 0.001$) levels.

Our findings indicate preparation for and participation in marathon running may affect multiple physiological pathways, and further studies using a larger panel of biomarkers are required in order to define the role of biomarkers in these non-elite athletes. The mechanism of release and prognostic meaning of abnormal concentration of these biomarkers remains uncertain. In athletes completing the Boston Marathon, increase in NT-proBNP and TnT (non-highly sensitive) correlated with post-race diastolic dysfunction, increased pulmonary pressures, and right ventricular dysfunction [3]. On the other hand, in non-marathon settings, these biomarkers confer long-term increased risk for myocardial infarction and cardiovascular death in individuals without symptoms of cardiovascular disease [4]. Among our trained recreational athletes, NT-proBNP concentrations modestly rose and fell; however, the substantial percentage of those with elevated ST2 concentrations together with huge release of hs-TnT during the race (along with persistently high circulating levels post-race) is noteworthy and could indicate exercise-induced myocyte injury, possibly due to mechanisms such as volume or pressure overload, myocardial strain, or direct myocyte toxicity. To our knowledge, our data are pioneer regarding endurance sports-driven ST2 increases in amateur runners; whether such release might be responsible for long-term extracellular matrix remodeling and, ultimately, adverse ventricular remodeling remains unclear. Ultimately, ST2 origin in endurance sports is unclear. Besides the heart, ST2 may have arisen from the microvasculature as endothelial cells are known to release ST2, or the lungs, as pulmonary sources of ST2 are also possible, or healing skeletal muscle may also be a source in marathon runners. Further research is needed to better understand ST2 pathobiology in endurance sports.

Marathon running and other endurance sports are increasingly popular, raising concerns about sports-driven adverse cardiac injury. More data and long-term follow-up studies in non-elite and elite athletes are needed. Nevertheless, excessive elaboration of cardiac biomarkers may emerge as Philippides surrogates, named for the Greek messenger who experienced sudden death after running more than 175 miles in 2 days.

Compliance with Ethical Standards

Funding No funding.

Conflict of Interest AB-G, JLJ, and JL have participated in lectures from Roche and Critical Diagnostics. PL has no disclosures. AB-G has received honoraria for lectures from Roche Diagnostics and Critical Diagnostics. AB-G has received grants from Roche Diagnostics.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Chapter 3: Salivary immunity and lower respiratory tract infections in non-elite marathon runners

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RESEARCH ARTICLE

Salivary immunity and lower respiratory tract infections in non-elite marathon runners

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Abstract

Rationale

Respiratory infections are common after strenuous exercise, when salivary immunity may be altered. We aim to investigate changes in salivary immunity after a marathon and its relationship with lower respiratory tract infections (LRTI) in healthy non-elite marathon runners.

Methods

Forty seven healthy marathon runners (28 males and 19 females) who completed the 42.195 km of the 2016 Barcelona marathon were studied. Saliva and blood samples were collected the day before the marathon and two days after the end of the race. Salivary IgA, antimicrobial proteins (lactoferrin, lysozyme) and chemokines (Groα, Groβ, MCP-1) were determined using ELISA kits in saliva supernatant. Blood biochemistry and haemogram were analyzed in all participants. The presence of LRTI was considered in those runners who reported infectious lower respiratory tract symptoms during a minimum of 3 consecutive days in the 2 weeks after the race.

Results

Eight participants (17%) presented a LRTI during the 2 weeks of follow-up. Higher lysozyme levels were detected after the race in runners with LRTI when compared with those without infection. A decrease in salivary lysozyme, Groα and Groβ levels after the race were observed in those runners who did not develop a LRTI when compared to basal levels. Salivary Groα levels correlated with basophil blood counts, and salivary lysozyme levels correlated with leukocyte blood counts.

Conclusions

LRTI are common after a marathon race in non-elite healthy runners. Changes in salivary antimicrobial proteins and chemokines are related to the presence of LRTI and correlate

with systemic defense cells, which suggest an important role of salivary immunity in the development of LRTI in non-elite marathon runners.

Introduction

The number of non-elite runners participating in marathons had increased dramatically in the last ten years [1]. Several studies have demonstrated that marathons and ultramarathons may alter immune function and increase the risk of respiratory tract infections [2–4]. Within two weeks of completing such strenuous exercise, the risk of infection increased 100% to 500% [5] and around 25% of finishers reported respiratory symptoms [3].

Salivary Immunoglobulin A (sIgA) is the most common antibody on the mucosal surface. Different studies have analyzed changes in sIgA following strenuous exercises and their potential relationship with respiratory symptoms, with conflicting results [6–8]. Other studies have suggested that immune factors present in mucosal secretion, including antimicrobial peptides (AMPs) and immune mediators such as chemokines, changed after prolonged running. An increase in salivary lactoferrin, one of the most abundant AMPs, has been described in participants in a 50 km ultramarathon [9]. Furthermore, chemokines such as Growth-Regulated Oncogene-alpha (Groα or CXCL1), growth-Regulated Oncogene-beta (Groβ) and monocyte chemoattractant protein 1 (MCP-1) have demonstrated an important role in the regulation of local immunity against infections by contributing to the tissue infiltration of leukocytes [10]. Experimental studies showed that serum levels of these inflammatory mediators increased markedly in response to exercise in mice [11]. However, limited data regarding salivary IgA, AMPs, chemokines and the presence of lower respiratory tract infections (LRTI) in marathon runners are available.

We hypothesized that non-elite marathon runner who experienced changes in their salivary immunity would be more prone to developing a respiratory infection. Therefore, our aim was to determine salivary levels of IgA, AMP and inflammatory markers before and after a marathon and their relationship with the development of LRTI.

Materials and methods

Subjects

Forty seven healthy non-elite marathon runners (28 males and 19 females) completed the 42.195 km of the 2016 Barcelona Marathon. Mean finishing time was 3 hours and 38 min (±41 min). Study participants were recruited using an announcement in the marathon newsletter that all marathon runners received two weeks prior to the race. Participation was voluntary in all cases. The study was approved by the local ethics committee "Comité Ètic de Investigació Clínica de la Fundació de Gestió Sanitaria del Hospital de la Santa Creu i Sant Pau de Barcelona", project number IIBSP-SUMMIT-2016-02. All participants gave signed informed consent.

All runners were asked to report any incidence of respiratory symptoms 2 weeks before and after the race. Runners who reported respiratory symptoms 2 weeks before marathon were excluded from the study. LRTI was defined according the clinical definition proposed by the European Respiratory Society and European Society for Clinical Microbiology and Infections disease [12]: acute illness (present for 21 days or less) characterized by cough as the main symptom, with at least one other respiratory tract symptom (sputum production, dyspnea, wheeze or chest discomfort/pain) and no alternative explanation. In addition, all runners had

to report at least one determination of fever (≥38˚C) and duration of symptoms must be higher than 3 days in order to exclude those symptoms induced only by strenuous exercise. All runners with suspected LRTI were follow-up until a breakdown of symptoms was reported.

Saliva and blood collection

Saliva and blood samples were collected one day before the marathon and two days after the end of the race. Saliva was collected into clean, sterile tub, maintained at 4˚C before centrifugation at 9500xg for 10 minutes at 4˚C. Supernatant was aspirated and 3 different aliquots were frozen at -20˚C until use. Three 10 mL blood samples were obtained from the antecubital vein and were transferred immediately (in less than 2 hours) to the laboratory for analysis.

Saliva analysis

The saliva samples were then thawed and total protein saliva was quantified using the Qubit protein Assay Kit (Thermofisher Scientific). Salivary IgA (Human IgA Platinum ELISA, ebioscience, Affymetrix, Santa Clare, CA), lactoferrin, lysozyme (AssayPro, St. Charles, MO, USA), Groα, Groβ and MCP-1 (Elabscience, Houston, Texas) were measured by ELISA according to the manufacturer's instructions. The limit of detection for IgA was: 1.6 ng/ml; lactoferrin: 0.625 ng/ml; lysozyme: 0.0781 ng/ml; Groα: 15.63 pg/ml, Groβ: 15.63 pg/ml and MCP-1: 15.63 pg/ml. Data were expressed as the concentration of each molecule relative to total saliva protein concentration [13]. Those determinations of relative salivary IgA concentration with a ratio higher than 1 were excluded of the study due to possible contaminations [14]. All determinations were performed in triplicate.

Blood analysis

Blood samples were centrifuged at 800xg at 4˚C for 10 min in a bench centrifuge. Supernatant (plasma) was aliquoted and stored on dry ice until all samples were frozen at -80˚C. Sodium and potassium plasma concentrations were determined using ion selective electrodes with an ionized sodium/potassium analyzer KNA1 (Radiometer, Copenhagen, Denmark). Creatinine and urea were measured using an AU5800 analyzer (Beckman Coulter, Hospitalet de Llobregat, Spain). C-reactive protein was determined using the AU-5800 Chemistry Analyzer (Bekman Coulter, Miami, FL, USA). Complete blood counts were performed with the Unicel DxH800 automated hematology analyzer (Beckman Coulter, Miami, FL, USA).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5 software. The Kolmogorov-Smirnov test was applied to test the normal distribution of the data. All variables with a normal distribution were reported as mean ± standard deviation (sd). T-test and paired t-test respectively were used for the comparison of independent and related variables with Gaussian distribution. The Wilcoxon test and Mann Whitney test were used for the comparison of related variables and for the comparison of independent variables respectively with non-normally distributed data. Pearson's and Spearman's coefficients respectively were used to correlate changes between normal and non-normal distributed variables. Chi-square tests were used for the comparison of frequencies. P values less than 0.05 were considered significant.

Results

Study participants

Table 1 shows demographic and laboratory test results prior to the marathon for all participants. Median age was 30 years old, median years of training were around 10 and training hours per week were around 6. All laboratory tests performed were in the normal range.

No differences between males and females were found in baseline characteristics (data not shown), except for Body Mass Index (BMI), which was higher in males than in females (23.6 ±2.1 vs 20.7 ± 1.4, p = 0.01).

Lower respiratory tract infections

Eight participants (17%) reported a LRTI during follow-up. No differences in demographics and laboratory findings between infected and non-infected participants were found prior to the marathon (Table 1). All runners with LRTI experienced a breakdown of the symptoms before 14 days of their onset and were fully recovered within one month after the race.

Salivary IgA

No differences before and after the marathon were found in total salivary protein concentration (2.34±1.16 mg/ml vs 2.37±0.97 mg/ml, respectively) nor in salivary IgA levels normalized to total salivary protein (0.39±0.25 vs 0.35±0.24) (Fig 1A). In addition, no differences in IgA levels before and after the race were found between infected and non-infected runners (Fig 1B), and no differences were observed between male and females before marathon in salivary IgA/protein.

Table 1. Demographic and biochemical charcateristics from marathon runners.

	All runners	Non LRTI	LRTI	p value
Sex (M/F)	28/19	21/18	7/1	NS
Age	39.0±7.1	39.1±7.4	38.6±5.6	NS
BMI	22.6±2.4	22.3±2.3	23.8±2.8	NS
Years training	9.7±9.3	10.2±10.0	7.4±4.0	NS
Hours training/week	6.5±3.7	6.5±3.9	6.6±2.6	NS
Glucose (mg/dl)	84.7±14.4	85.5±15.4	81.0±7.9	NS
Na (mEq/l)	139.3±1.6	139.2±1.4	139.8±2.5	NS
K (mEq/l)	4.0±1.6	4.0±0.2	4.0±0.3	NS
Leukocytes %	6.9±1.6	6.8±1.4	7.6±2.2	NS
% Lymphocytes	34.7±7.5	34.8±6.5	34.1±11.9	NS
% Monocytes	8.3±2.7	8.5±2.9	7.7±1.3	NS
% Neutrophils	53.9±8.5	53.5±7.5	55.8±12.6	NS
% Eosinophils	2.3±1.7	2.4±1.7	1.7±1.5	NS
% Basophils	0.6±0.4	0.7±0.4	0.4±0.1	NS
Hb (g/dl)	14.1±1.1	14.5±0.9	14.0±1.2	NS
Hematocrit (%)	41.8±3.3	41.6±3.4	42.8±2.9	NS
Platelets ($x10^9$/l)	215.8±50.3	214±48	220±62	NS
CRP (mg/dl)	1.3±2.2	1.4±2.4	1.0±0.5	NS

BMI, Body Mass Index; Hb, hemoglobin; CRP, C-reactive protein.

LRTI: Lower Respiratory Tract Infection

NS, non significant

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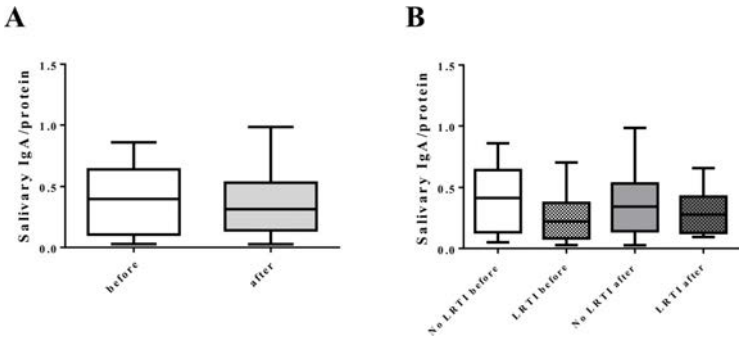

Fig 1. Salivary sIgA from marathon non-elite runners. Salivary sIgA was determined by ELISA as described in Materials and Methods. (A) sIgA content before and after marathon. (B) sIgA content before and after marathon based on the development of LRTI after marathon. Results were showed as the concentration of sIgA relative to total protein content. P values were determined by paired non parametric Wilcoxon t-test.

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Salivary antimicrobial proteins

No differences before and after the race were observed in salivary lactoferrin (0.013±0.01 vs 0.010±0.007) and salivary lysozyme levels (0.0094±0.007 vs 0.0078±0.007). Furthermore, no differences between the sexes were observed before the marathon.

When runners with and without LRTI were compared, we observed no differences in salivary lactoferrin levels before and after the race. However, those runners who developed a LRTI showed higher levels of salivary lysozyme after the race compared to those who did not develop it (0.012±0.01 vs 0.006±0.004, p = 0.02). In addition, a decrease in lysozyme levels was observed in runners who did not develop LRTI when levels before and after the race were compared (0.010±0.007 vs 0.006±0.004, p = 0.003) (Fig 2).

Salivary chemokines

No differences before and after the race were observed regarding salivary Groα Groβ and MCP-1. However, runners who did not develop LRTI showed a significant decrease in salivary Groα and Groβ levels after the race (Groα, 0.37±0.15 vs 0.30±0.14, p = 0.02; Groβ 0.47±0.17 vs 0.37±0.18, p = 0.03) (Fig 3A and 3B). No differences in MCP-1 levels were found.

Systemic correlations

Before the race, salivary lactoferrin levels had a weak but statistical significant negative relationship with blood lymphocyte counts (r = -0.13, p = 0.04) and salivary Groα had a weak positive correlation with the percentage of blood basophils (r = 0.22, p = 0.03) (Fig 4A and 4B).

After the race, salivary lysozyme correlated positively with the absolute number of blood leukocytes (r = 0.26, p = 0.006) (Fig 4C).

Discussion

In our study we observed that LRTI is common in non-elite runners following a marathon and we demonstrated that runners who developed a LRTI had different immunological profile in their saliva. Specifically, a decrease in salivary lysozyme, Groα and Groβ levels was observed in those runners who did not develop a LRTI when levels before and after the race were compared. In addition, higher levels of lysozyme were detected after the race in runners with LRTI

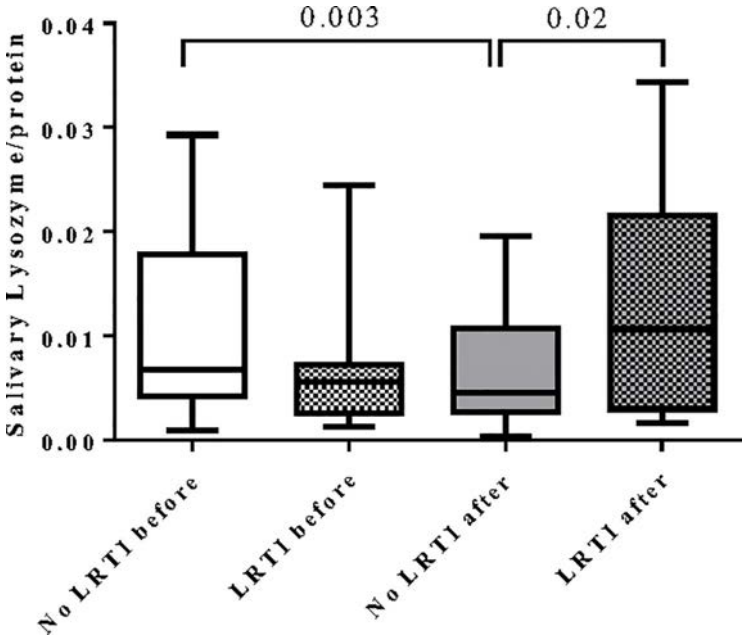

Fig 2. Salivary lysozyme from marathon non-elite runners before and after the marathon. Results were showed as the concentration of lysozyme relative to total protein content. P values were determined by unpaired t-test and paired Wilcoxon test.

https://doi.org/10.1371/journal.pone.0206059.g002

compared with those without infection. These findings suggest that immunological status may be important for the development of a LRTI after a marathon.

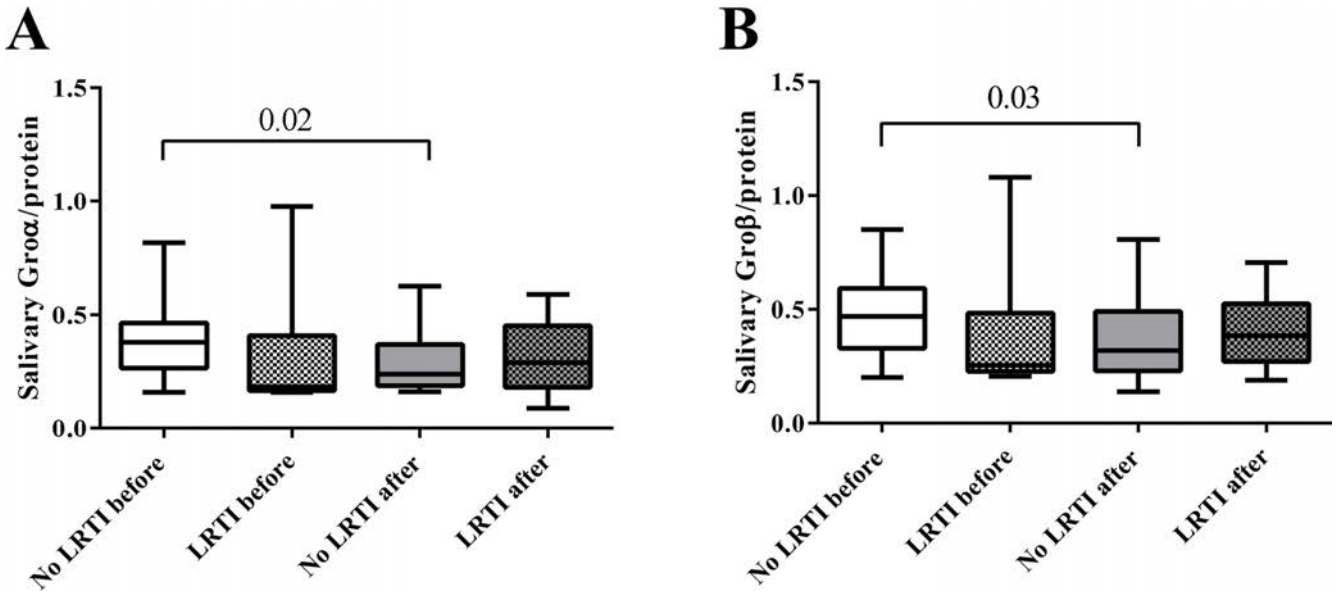

Fig 3. Salivary Groα and Groβ. Salivary (A) Groα and (B) Groβ were determined by ELISA as described in Materials and Methods before and after marathon. P values were determined by unpaired Mann Whitney t-test and paired Wilcoxon test.

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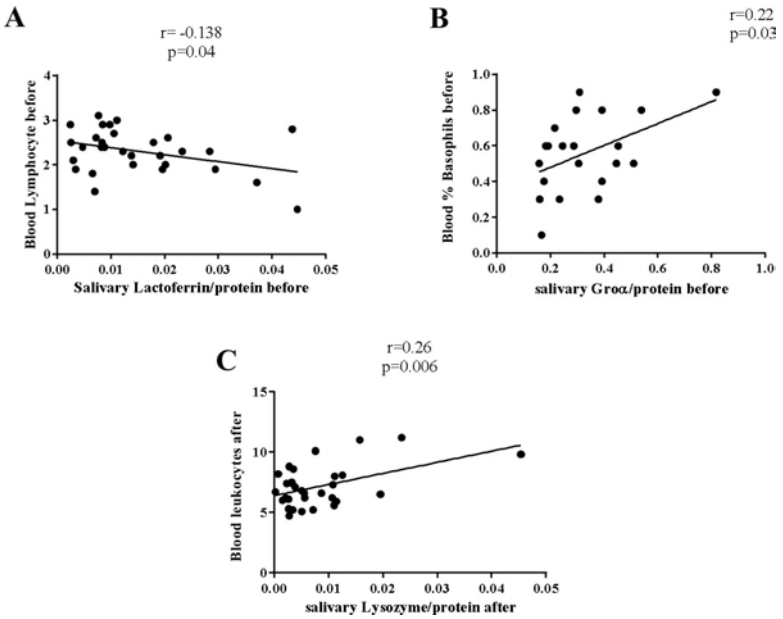

Fig 4. Correlation of salivary lactoferrin, Groα and lysozyme with systemic haematological parameters. (A) Negative correlation between salivary lactoferrin with blood lymphocytes, (B) positive correlation between salivary Groα and blood basophils and (C) positive correlation between salivary lysozyme and blood leukocytes, were found. Pearson's and Spearman's coefficients were respectively used to correlate changes between normal and between non-normal distributed variables.

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Several data have been reported reflecting the importance of the immune system in the development of LRTI after strenuous exercise. We observed that 17% of marathon runners develop LRTI symptoms within 2 weeks after marathon, which is concordant to other studies [7,15]. Although the effect of close proximity between runners during the race cannot be ruled out, extreme effort has been shown to alter immune defenses by increasing inflammation in the respiratory tract [16]. Different studies have postulated that a combination of several immune, biochemical and hematological parameters participate in the severe stress on the body resulting from such exercise and that they may be associated with an increased susceptibility to developing infections [17]. Salivary IgA (sIgA) is one of the most widely-studied parameters [7]. Some studies have pointed to the usefulness of sIgA as a noninvasive biomarker of mucosal immunity and LRTI risk [18,19]. However, other studies have suggested that reductions in sIgA cannot be solely responsible for the decline in immune function that may lead to LRTI [20]. In our cohort of runners, we did not observe differences in sIgA as other authors previously showed [21]. We observed that sIgA represented about 40–60% of total salivary protein, which is concordant with previous studies [22,23] but higher than other ones [24]. Most of the studies were carried out few hours after exercise [25,26] and we wanted to evaluate two days after finishing the marathon, since few data regarding the recovery period and its influence on the development of LRTI are available [7].

Salivary antimicrobial proteins (sAMPs) such as lysozyme and lactoferrin protect the respiratory tract from invading microorganisms and have been linked with an increased infection risk in athletes [27]. In our cohort of runners, we observed no differences in the levels of saliva lysozyme before the race. However, runners who did not develop LRTI showed a decrease in lysozyme after the marathon. In contrast, those runners who developed a LRTI showed higher

levels of salivary lysozyme after the race compared to those runners who did not develop LRTI. Our findings are in line with other studies showing a decrease in salivary lysozyme immediately after an ultra-marathon, though this decrease was not related to the presence of LRTI [5]. However, other studies did not observe differences in salivary lysozyme levels after a 50 km race [25]. These discrepancies could be the consequence of different levels of effort during exercise. Interestingly we observed an inverse relation between salivary lactoferrin and blood lymphocyte counts before the race, which may be explained by the mobilization of neutrophils to maintain blood homeostasis. Previously, Inoue H et al. [28] described an immediate increase in serum lactoferrin concentrations immediately after running exercise and serum lactoferrin may play an antibacterial role in host defenses before the mobilization of neutrophils into the circulating pool. We also observed a positive correlation between blood leukocyte counts and the content of saliva lysozyme after the marathon. It has been observed that exercise sessions in both humans and animal models resulted in the stimulation of neutrophil degranulation releasing lysozyme from blood neutrophils [29]. All of these findings suggested that systemic alterations may affect the content of saliva lactoferrin and lysozyme in runners and may contribute to the development of LRTI after races. Nevertheless, we cannot exclude a process of dehydration during the marathon, since it has been demonstrated that dehydration decreases saliva antimicrobial proteins [30,31]. Dehydration can therefore affect the content of saliva AMPs, and further studies are needed to better elucidate its importance, though we did not observe differences in protein content before and after the race.

Different chemokines have demonstrated a potential role in the regulation of immune response during exercise [32]. We also found that Groα and Groβ, which are key components in the attraction of neutrophils to the site of inflammation, decreased after the race in runners who did not develop a LRTI. It has been described that daily moderate exercise suppressed Groα [33] and, in experimental models, exercise down regulated multiple inflammatory cytokines and chemokines including Groα and MCP-1 [34,35] and had a systemic anti-inflammatory effect, reducing Groα, Groβ and MCP-1 [36,37]. These findings were observed in runners who did not develop LRTI, suggesting that they had a better inflammatory regulation response that may protect against infection. Therefore, athletic performance is both a stress factor and an adaptive response to exercise that can be modulated by training, reduce inflammation and help to prevent disease. Further studies are needed to better understand the relationship between salivary immunity and systemic inflammation as a key factor in the development of LRTI after exercise.

Interestingly, we observed a positive correlation between blood basophils and salivary Groα level before the race. Sastre B et al, identified basophils as a new player in the status of bronchial inflammation in athletes [38]. We observed that non LRTI runners had a higher percentage of systemic basophils, although differences were not statistically significant.

Our study has several limitations. First, due to the small number of runners included, the results should be validated in other studies before generalizing them. Second, since some data reported the effect of feeding in post exercise saliva AMPs proteins [39] we cannot exclude feeding related affectations in the determinations of AMPs in our cohort of runners. Third, levels of leukocyte subpopulations after the race were measured 48 hours after the marathon and other determinations at 7 or 14 days would be helpful to better characterize systemic response in marathon runners, and it should be an important point to be taken into account in further studies. And fourth, infectious agents that caused LRTI are not described due to the absence of microbiological studies. Further studies including bacterial and viral determinations would be very helpful to clarify this important issue.

In conclusion, non-elite runners who developed a LRTI after a marathon showed a differential profile of saliva IgA, AMPs and chemokines compared to those runners who did not

develop infection. Therefore, exercise training and post-marathon recovery would be important in the immunological profile and the risk of developing a LRTI. Further studies are needed to better understand the underlying mechanisms and the impact of salivary immunity and its regulation in the prevention and development of LRTI in non-elite marathon runners.

Supporting information

S1 File. PONE-D-18-11926R3_Data.xlsx.
(XLSX)

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Chapter 4: Effects of a polysaccharide-based multi-ingredient supplement on salivary immunity in non-elite marathon runners

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RESEARCH ARTICLE Open Access

Effects of a polysaccharide-based multi-ingredient supplement on salivary immunity in non-elite marathon runners

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Abstract

Background: Extreme exercise may alter the innate immune system. Glycans are involved in several biological processes including immune system regulation. However, limited data regarding the impact of glycan supplementation on immunological parameters after strenuous exercise are available. We aimed to determine the impact of a standardized polysaccharide-based multi-ingredient supplement, Advanced Ambrotose© complex powder (AA) on salivary secretory Immunoglobulin A (sIgA) and pro- and anti-inflammatory protein levels before and after a marathon in non-elite runners.

Methods: Forty-one male marathon runners who completed the 42.195 km of the 2016 Barcelona marathon were randomly assigned to two study groups. Of them, $n = 20$ (48%) received the AA supplement for 15 days prior the race (AA group) and $n = 21$ (52%) did not receive any AA supplement (non-AA group). Saliva and blood samples were collected the day before the marathon and two days after the end of the race. Salivary IgA, pro-inflammatory chemokines (Gro-alpha, Gro-beta, MCP-1) and anti-inflammatory proteins (Angiogenin, ACRP, Siglec 5) were determined using commercially ELISA kits in saliva supernatant. Biochemical parameters, including C-reactive protein, cardiac biomarkers, and blood hemogram were also evaluated.

Results: Marathon runners who did not receive the AA supplement experienced a decrease of salivary sIgA and pro-inflammatory chemokines (Gro-alpha and Gro-beta) after the race, while runners with AA supplementation showed lower levels of anti-inflammatory chemokines (Angiogenin). Gro-alpha and Gro-beta salivary levels were lower before the race in the AA group and correlated with blood leukocytes and platelets.

Conclusions: Changes in salivary sIgA and inflammatory chemokines, especially Gro-alfa and Gro-beta, were observed in marathon runners supplemented with AA prior to the race. These findings suggested that AA may have a positive effect on immune response after a strenuous exercise.

Keywords: Glycans, Dietary plant-derived polydisperse polysaccharide supplementation, Salivary sIgA, Inflammatory chemokines, Marathon, Strenuous exercise

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Background

In recent years, there has been a significant increase of participants in ultra-endurance races such as marathons and ultramarathons. In the United States alone, marathon runners have increased from 25,000 in 1976 to almost 503,000 in 2010 [18]. Competing in very strenuous events imposes severe metabolic stress and causes acute responses that may negatively alter the immune system [17]. A high level of physical demand during such events induces a wide range of metabolic changes and causes micro-injuries in the muscles and other tissues. This physical demand increases the migration of white blood cells to the sites of injury, and induces acute phase inflammatory reactions [5, 16, 28]. The local response to tissue injury involves the production of a large number of acute phase proteins and cytokines related to innate immunity such as secretory Immunoglobulin A (sIgA) or inflammatory chemokines [9, 30]. Secretory Immunoglobulin A (sIgA) is a first line of defense against external agents, serving as a noninvasive biomarker of mucosal immunity [10]. Several studies have shown significant sIgA changes following strenuous exercise [3, 19, 24]. Other important immune factors present in mucosal secretion also change after prolonged running [6, 20, 24]. These changes include: pro-inflammatory chemokines, such as Gro-alpha (neutrophil-activating protein 3 and melanoma growth stimulating activity), Gro-beta (macrophage inflammatory protein, secreted by monocytes and macrophages and chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells) and MCP-1 (to recruit monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection); anti-inflammatory proteins such as Angiogenin (associated with altered normality through angiogenesis and through activating gene expression that suppresses apoptosis), Adipokine ACRP30 (cell signaling protein secreted by adipose tissue involved in regulating glucose levels as well as fatty acid breakdown) and Siglec5 (Sialic acid-binding Ig-like lectin 5).

Glycans (a generic term for any sugar or assembly of sugars, in free form or attached to another molecule) are directly involved in physiology [31]. Glycans are involved in inflammation and immune system activation [11]. A standardized polysaccharide-based multi-ingredient supplement including glycans (Advanced Ambrotose© complex powder (AA)) may produce a significant overall shift towards an increased sialylation of serum glycoproteins. Sialylation changes can have a key role in many aspects of the immune response [2]. It is therefore not surprising that one of the physiological effects of these polysaccharides is the immunomodulation through the glycosylation of proteins [15]. AA is a saccharide supplement containing a standardized combination of plant polysaccharides (a source of mannose, galactose, fucose, xylose, glucose, n-acetyl-glucosamine, n-acetyl-neuraminic acid, and n-acetyl-galactosamine) that may regulate immunity. In controlled human trials, polysaccharide intake enhanced the immune system biomarkers in the blood of healthy adults [22]. The effect of dietary supplements with polysaccharides on retired professional football players supported and optimized their quality of life [25]. However, no data regarding the impact of AA supplementation on healthy marathon runners performing strenuous physical activity is available.

Our hypothesis is that glycan supplementations before strenuous physical activity enhances immune function and balances pro-inflammatory and anti-inflammatory proteins. Therefore, the aim of this study is to determine the impact of AA in the levels of sIgA, pro-inflammatory chemokines and anti-inflammatory proteins before and after running a marathon in non-elite marathoners.

Methods

Participants

This study is a part of the SUMMIT project (Health in Ultra-Marathons and their Limits), whose objective is to evaluate the behavior of certain clinical parameters in different races and was approved by an institutional review board (IIBSP-SUMMIT-2016-02). In this study, 41 male non-elite runners of the Barcelona Marathon 2016 participated and they gave signed informed consent.

All runners were weighed on race morning within 2 h of race start in racing attire with running shoes and immediately after completing the race using the same scale Jata 565 model. Later the body mass index (BMI) was calculated using the formula BMI = weight (kg) / height (m^2). The mean age was 39.3 ± 9.2 years old.

Study groups

Twenty participants (48%) received AA supplementation prior to the race (AA group), while 21 participants (52%) did not receive the AA supplement prior to the race (non-AA group). AA was administrated at dose of 8 g/day (with Arabinolactan, *Aloe Vera* extract, rice starch, ghatti gum, gum Tragacanth, Glucosamine HCl, Wakame algae extract) for 15 days before the marathon. The 41 athletes were selected for being homogeneous in age, weekly training hours, years of training, weight and height (Table 1), and were randomly assigned into the two study groups.

Saliva and blood collection

Saliva samples were collected 48 h before the marathon (d0 samples) and two days after the end of the race (d2 samples). Saliva was collected into clean, sterile tubes, maintained at 4 °C before centrifugation at 9500×g 10 min at 4 °C. Supernatant was aspirated and 3 different aliquots were frozen at − 20 °C, until evaluated.

Table 1 Participant characteristics, anthropometric and training data before the marathon

Baseline characteristics	Total cohort	Non-AA group	AA group	*p*-value
Number of runners	41	21	20	
Age (years)	39.3 ± 9.2	39.3 ± 12.8	39.2 ± 4.9	0.717
Weight (Kg)	77.0 ± 11.0	74.0 ± 12.0	78.5 ± 12.0	0.208
Height (cm)	178.0 ± 8.0	178.0 ± 7.0	179.5 ± 8.0	0.142
BMI (kg/m^2)	24.1 ± 3.4	23.8 ± 3.0	24.2 ± 3.7	0.696
Training (years)	8.0 ± 8.5	7.0 ± 6.5	9.0 ± 12.3	0.537
Training (hours/week)	7.0 ± 5.3	6.0 ± 4.5	7.0 ± 4.9	0.106
Loss weight (%)	3.1 ± 1.8	3.1 ± 2.9	3.1 ± 1.7	0.276
Race time	3.5 ± 0.5	3.4 ± 0.6	3.6 ± 0.5	0.265

AA group: Participants received Advanced Ambrotose Complex Powder (AA) supplementation prior the race
Non-AA group: Participants did not received any AA supplement prior the race
BMI: body mass index
Values are reported as mean ± SD for the quantitative and in percentage for the categorical. ANOVA test was used for parametric variables; Kruskal Wallis test was used for non parametric variables; Chi-Square Pearson for categorical variables

Three 10 mL blood samples were obtained from the antecubital vein 48 h before the marathon (d0 samples), at completion (d1 samples), and 48 h after the race (d2 samples). After the marathon, samples (d1) and weights were obtained within the 10 min interval after completing the race and before drinking any fluid or emptying the bladder.

Saliva analysis

Saliva samples were thawed and total saliva protein was quantified using Qubit protein Assay Kit (molecular probes Life Technologies). Salivary IgA (Human IgA, Platinum ELISA, ebioscience, Affymetrix, Santa Clare, CA), Lactoferrin, Lysozyme (AssayPro, St, Charles, MO, USA), Gro-alpha/CXCL1, Gro-beta/CXCL2 and MCP-1 (Elabscience, Houston, Texas) were measured by ELISA according to manufacturer's instructions. Limits of detection were for IgA: 1.6 ng/ml; lactoferrin: 0.625 ng/ml; lysozyme: 0.0781 ng/ml; Gro-alpha/CXCL1: 15.63 pg/ml, Gro-beta/CXCL2: 15.63 pg/ml, MCP-1: 15.63 pg/ml, Angiogenin: 1.64 pg/ml, ARCP30: 24.69 pg/ml and Siglec 5: 6.86 pg/ml. The data was expressed as concentration of each protein relative to total saliva protein concentration.

Blood analysis

The complete blood counts were performed on the Unicel DxH800 automated hematology analyzer (Beckman Coulter, Miami, FL, USA). N-terminal pro-B-type natriuretic peptide (NT-proBNP) was quantified in whole EDTA blood using the AQT90 FLEX immunoassay (Radiometer Medical, Copenhagen, Denmark). Serum creatine kinase (CK) and C-reactive protein (CRP) were determined using the AU-5800 Chemistry Analyzer (Beckman Coulter). Troponin T was measured from serum, using a High Sensitive Troponin-T assay in a Cobas e601 platform (Roche Diagnostics, Barcelona, Spain). ST2 was measured from serum samples using a high-sensitivity sandwich monoclonal immunoassay (Pressage® ST2 assay, Critical Diagnostics, San Diego, CA, USA).

Statistical analysis

The Kolmogorov-Smirnov test was applied to test the normal distribution of the data. All variables, with a normal distribution, were reported as mean ± standard deviation (SD). The rest of the variables were reported as median (interquartile rank) (IQR). T-test and paired t-test were respectively used for the comparison of independent and related variables with normal distribution. Wilcoxon test was used for the comparison of related variables with non-normally distributed data. Pearson's and Spearman's coefficients were respectively used to correlate changes between normal and between non-normal distributed variables. ANOVA and Kruskal-Wallis test were respectively used for comparative analyses of multiple normal and non-normal distributed data. Chi-square tests were used for the comparison of frequencies. *P* values less than 0.05 were considered significant.

Results

Study participants

Forty-one male non-elite marathon runners completed the 42.195 km of the 2016 Barcelona Marathon. Mean finishing time was 3 h and 28 min ± 0. 41 h. Median (interquartile range) age was 39 ± 9 years old, with a weight of 77 ± 11 kg, height of 178 ± 8 cm and Body Mass Index (BMI) of 24.1 ± 3.4. Median training years were 8 ± 8, with a median of 7 ± 5 h per week.

No significant differences in age, weight, height, and training were found between runners who received AA supplementation and those who did not (Table 1).

Plasma and blood measurements

C-reactive protein increased significantly 48 h after the marathon (d2), although no significant differences

between AA and non-AA groups were found. Similarly, all evaluated cardiac (Hs-TnT, St2 and NTproBNP) and muscle damage (CK) biomarkers increased after the race, but no significant differences between participants in either group were found (Table 2).

Hemoglobin, hematocrit, erythrocytes and platelets counts did not change after the race (d1), with no differences between groups. However, a marked increase in leukocyte counts per L was found in both groups after the race (d1) and participants who received AA supplementation for 15 days prior to the race experienced a lower increase compared to those without the AA supplementation (13.2 ± 4.6 vs $16.1 \pm 4.4 \times 10^9$/L, $p = 0.03$) (Table 2).

Salivary IgA

No differences in salivary IgA levels normalized to total salivary protein before (d0) and 48 h after the race (d2) were found (0.47 ± 0.44 vs 0.37 ± 0.29, $p = 0.6$). However, when participants with and without AA supplementation were compared, a decrease of sIgA 48 h after the race (d2) was observed in the non-AA group (0.55 ± 0.37 vs 0.31 ± 0.35, $p = 0.01$), while no differences

Table 2 Plasma and blood measurements before, after and 48 h after the marathon in 41 runners

Biochemical values	Total cohort	Non-AA group	AA group	*p*-value
CRP_d0 (mg/dL)	0.85 ± 1.18	0.60 ± 1.28	1.00 ± 1.23	0.683
CRP_d1 (mg/dL)	0.55 ± 0.85	0.45 ± 2.91	0.95 ± 2.48	0.606
CRP_d2 (mg/dL)	7.6 ± 4.65	6.80 ± 5.00	8.95 ± 4.25	0.359
CK_d0 (U/L)	170.5 ± 123.8	188.5 ± 172.3	136.0 ± 125.3	0.633
CK_d1 (U/L)	566.0 ± 282.3	566.0 ± 251.5	567.5 ± 396.3	0.958
CK_d2 (U/L)	764.5 ± 540.8	788.5 ± 787.8	650.0 ± 513.8	0.368
Hs-TnT_d0 (ng/L)	3.1 ± 2.7	2.9 ± 2.0	3.4 ± 5.0	0.407
Hs-TnT_d1 (ng/L)	49.6 ± 68.1	48.9 ± 68.8	49.9 ± 68.8	0.449
Hs-TnT_d2 (ng/L)	4.8 ± 6.3	4.9 ± 7.2	4.6 ± 3.7	0.334
St2_d0 (ng/mL)	34.0 ± 14.8	34.7 ± 16.2	28.6 ± 16.0	0.394
St2_d1 (ng/mL)	54.9 ± 30.3	59.0 ± 40.5	43.3 ± 30.4	0.256
St2_d2 (ng/mL)	32.9 ± 20.5	37.7 ± 19.3	29.4 ± 17.1	0.059
NTproBNP_d0 (ng/L)	70.0 ± 0	70.0 ± 0	70.0 ± 0	0.336
NTproBNP_d1 (ng/L)	94.5 ± 70.5	100.5 ± 50.3	85.5 ± 221.3	0.392
NTproBNP_d2 (ng/L)	70.0 ± 0	70.0 ± 0	70.0 ± 0.5	0.845
Hemoglobin_d0 (g/L)	14.8 ± 1.3	14.8 ± 1.1	14.7 ± 1.6	0.350
Hemoglobin_d1 (g/L)	14.7 ± 1.1	14.8 ± 1.0	14.5 ± 0.3	0.173
Hemoglobin_d2 (g/L)	14.3 ± 0.7	14.4 ± 0.7	14.2 ± 0.9	0.170
Hematocrit_d0	43.7 ± 3.0	43.7 ± 3.1	43.8 ± 3.1	0.327
Hematocrit_d1	44.0 ± 4.2	44.3 ± 3.2	43.3 ± 3.4	0.132
Hematocrit_d2	42.7 ± 2.2	42.8 ± 1.8	42.2 ± 3.7	0.114
Erythrocytes_d0 (10Exp12/L)	4.9 ± 0.6	4.9 ± 0.4	5.1 ± 0.7	0.903
Erythrocytes_d1 (10Exp12/L)	5.0 ± 0.5	5.0 ± 0.4	5.0 ± 0.8	0.586
Erythrocytes_d2 (10Exp12/L)	4.8 ± 0.4	4.8 ± 0.3	4.9 ± 0.5	0.532
Platelets_d0 (10Exp9/L)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.063
Platelets_d1 (10Exp9/L)	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.852
Platelets_d2 (10Exp9/L)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.555
Leukocyte_d0 (10^9/L)	6.5 ± 1.9	6.4 ± 1.3	6.5 ± 3.2	0.860
Leukocyte_d1 (10^9/L)	12.2 ± 5.3	16.1 ± 4.4	13.2 ± 4.6	0.032
Leukocyte_d2 (10^9/L)	6.6 ± 1.3	6.6 ± 1.4	6.6 ± 1.8	0.217

AA group: Participants received Advanced Ambrotose Complex Powder (AA) supplementation prior the race
Non-AA group: Participants did not received any AA supplement prior the race
CK creatine kinase, *CRP* C-reactive protein, *HGB* hemoglobin, *Hs-TnT* high-sensitivity troponin T, *NT-proBNP* N-terminal pro-B-type natriuretic peptide
Values are reported as mean ± SD for the quantitative and in percentage for the categorical. ANOVA test was used for parametric variables; Kruskal Wallis test was used for non parametric variables; Chi-Square Pearson for categorical variables

before (d0) and 48 h after the race (d2) in the AA group were found (0.25 ± 0.33 vs 0.24 ± 0.32, $p = 0.5$) (Fig. 1).

Salivary pro inflammatory proteins

A decrease of pro-inflammatory salivary proteins such as Gro-alfa, Gro-beta and MCP-1 after the race was observed in all participants, although differences were not statistically significant (data not shown). Nevertheless, the decline in Gro-alpha (Fig. 2a) and Gro-beta (Fig. 2b) was statistically significant in those runners who did not receive the AA supplement (0.38 ± 0.20 vs 0.24 ± 0.18, $p = 0.02$ and 0.47 ± 0.26 vs 0.32 ± 0.26, $p = 0.03$), while differences were not observed in the AA group. Furthermore, basal levels of Gro-alpha and Gro-beta were significantly lower (p = 0.03) in the AA group at baseline before the marathon (d0) compared to the non-AA group (Fig. 2).

Salivary anti-inflammatory proteins

No statistically significant differences of salivary anti-inflammatory proteins such as ACRP, angiotensin, and Siglec 5 were observed before (d0) and 48 h after the marathon (d2) (data not shown). However, in those runners of the AA group, a significant decrease of Angiogenin 48 h after the race (d2) was observed (0.18 ± 0.08 vs 0.14 ± 0.07, $p = 0.04$), while differences were not observed in the non-AA group (0.3 ± 0.13 vs 0.17 ± 0.10, $p = 0.7$) (Fig. 3).

Systemic correlations

After the race, those runners who received the AA supplement (AA group) showed positive correlations between salivary Gro-alpha levels with leukocyte counts per L (r = 0.38, $p = 0.02$) and a tendency between Gro-beta salivary levels and blood platelet counts (r = 0.27, $p = 0.06$).

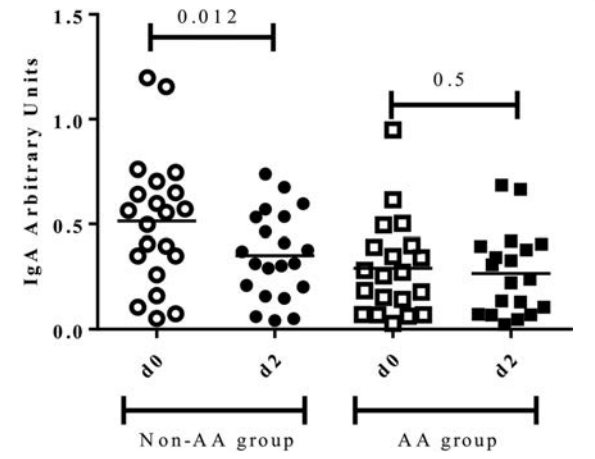

Fig. 1 Salivary IgA levels before (d0) and 48 h after the marathon (d2) between groups

These systemic correlations were not observed in the non-AA group (Fig. 4).

Discussion

In our study, we demonstrated significant changes in salivary biomarkers of immune function in healthy, non-elite athletes before and after a strenuous exercise like an asphalt marathon, after consuming a polysaccharide-based multi-ingredient supplement, AA, for 15 days prior to exercise. Specifically, a decrease in salivary sIgA, Gro-alfa and Gro-beta were observed after the marathon in those runners who did not receive AA supplement prior the race, while those runners who received the AA supplement showed a decrease in a salivary Angiogenin. Gro-alpha and Gro-beta salivary levels were lower before the race in the AA group and correlated with the counts of blood leukocytes and platelets. These findings suggest that AA supplementation produces changes in salivary immunity that may have a positive effect on immunity before and after a marathon.

sIgA is a key component of innate immunity and provides a first line of defense against pathogens at mucosal surfaces [8]. Numerous studies have assessed the saliva sIgA response to prolonged strenuous exercise to explore whether immunity may be temporarily compromised after the exercise. Most of the studies described a post-exercise decrease in sIgA, including Nordic skiers after a 50 km race [29] or trained cyclists cycling for more than 2 h on a stationary ergometer [32]. In marathon runners, a significant decrease of sIgA following a marathon race has been described [19], which was independent of gender, age or carbohydrate ingestion. In our study, runners with AA supplementation did not experience a significant decrease in sIgA after the marathon, which was observed in runners without the AA. These findings suggested that AA supplement enhanced immunity by avoiding the sIgA decrease after the race. Different studies have postulated that glycosylation plays an important role in the biosynthesis and biological activity of the proteins involved in antigen recognition, such as sIgA [23]. Further studies are needed to explore this pathway, which may be crucial in order to better understand the effect of strenuous exercise on mucosal immunity.

Supplementation with glycans can also result in salivary glycol-modifications of proteins. These modifications can regulate the synthesis and/or degradation of pro- and anti-inflammatory molecules participating in the immune response of runners to strenuous effort. Several inflammatory chemokines have demonstrated a potential role in the regulation of immune response during exercise [27]. In our study, we demonstrated that Gro-alfa and Gro-beta, two pro-inflammatory chemokines, involved in the attraction of neutrophils to the site of inflammation, decreased in saliva after the race in those runners who did not

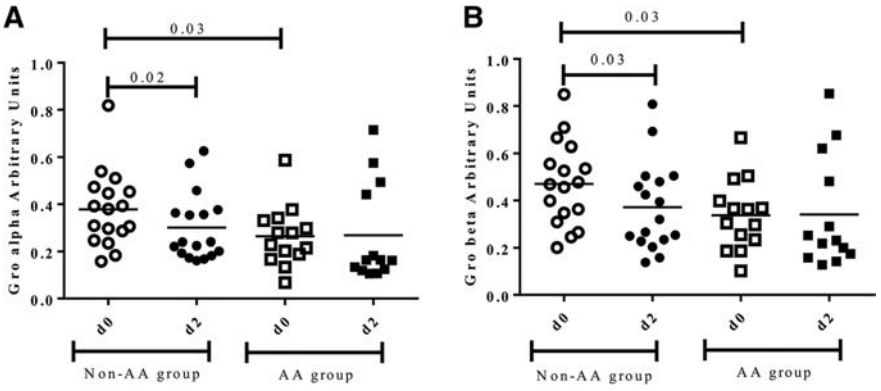

Fig. 2 Salivary pro inflammatory proteins, gro alpha (**a**) and gro beta (**b**) before (d0) and 48 h after the marathon (d2) between groups

receive AA. It has been described that moderate exercise suppressed Gro-alfa [4] and, in experimental models, exercise down-regulates multiple inflammatory cytokines [26], including Gro-alfa and Gro-beta, that results in a systemic anti-inflammatory effect [1, 13]. These findings were not observed in runners who received the AA supplement, suggesting that they had better immunoregulation. Runners with AA supplementation have also showed a decrease in salivary Angiogenin levels, which was not detected in runners without AA. It suggests that salivary pro-inflammatory proteins can be buffered with anti-inflammatory ones, especially those produced by runners supplemented with AA. In addition, it is important to note that after 15 days on AA, the baseline levels of Gro-alfa, Gro-beta and Angiogenin were different in runners with and without AA. Interpretations of all these salivary inflammatory changes are complex and require further more detailed studies.

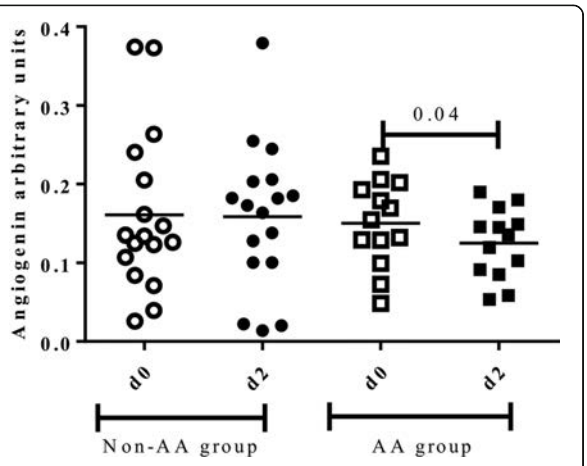

Fig. 3 Salivary anti-inflammatory protein Angiogenin before (d0) and 48 h after the marathon (d2) between groups

The systemic effect of glycans has been described in several studies [15]. Interestingly, it is postulated that glycans may regulate leukocyte activity [21]. In our study, runners who received the AA supplement experienced a lower increase in total blood leukocyte counts after the race compared to those runners without the AA supplement. Furthermore, a correlation between salivary levels of Gro-alpha with blood leukocyte counts and salivary Gro-beta levels with blood platelet counts were observed in the AA group. All in all, these findings suggested a relationship between salivary and systemic immunity, especially in those runners who received the AA supplement prior to the race. The association between high dietary protein during high-intensity training and reduction in respiratory symptoms in elite cyclists by restoring impairments in leukocyte trafficking has also been reported [35] The influence of ingredients other than glycans on the immune system has been reported. For example, brown seaweed [33], *Aloe vera* [12] and glucosamine [14] have antioxidant activity and anti-inflammatory effects. Arabinogalactan decreases the incidence of infectious episodes by improving serum-antigen specific IgG and IgE response to *Streptococcus pneumonia* [7]. Starch supplementation promotes the growth of commensal bacteria that may improve bowel health [34]. However, more work is needed to clarify the mechanism that may explain these important results.

Our study has limitations. First, we only have studied men, and we cannot exclude the effect of glycans in salivary immunity based on sex. Second, due to the small sample size of runners included, the results should be validated in further studies before generalizing them. Third, runners received the AA supplement for 15 days prior the race, and the effect of taking this supplement for a longer time may be different. Fourth, the influence of ingredients included in the AA supplements which are not glycans such as glucosamine and *Aloe Vera* on

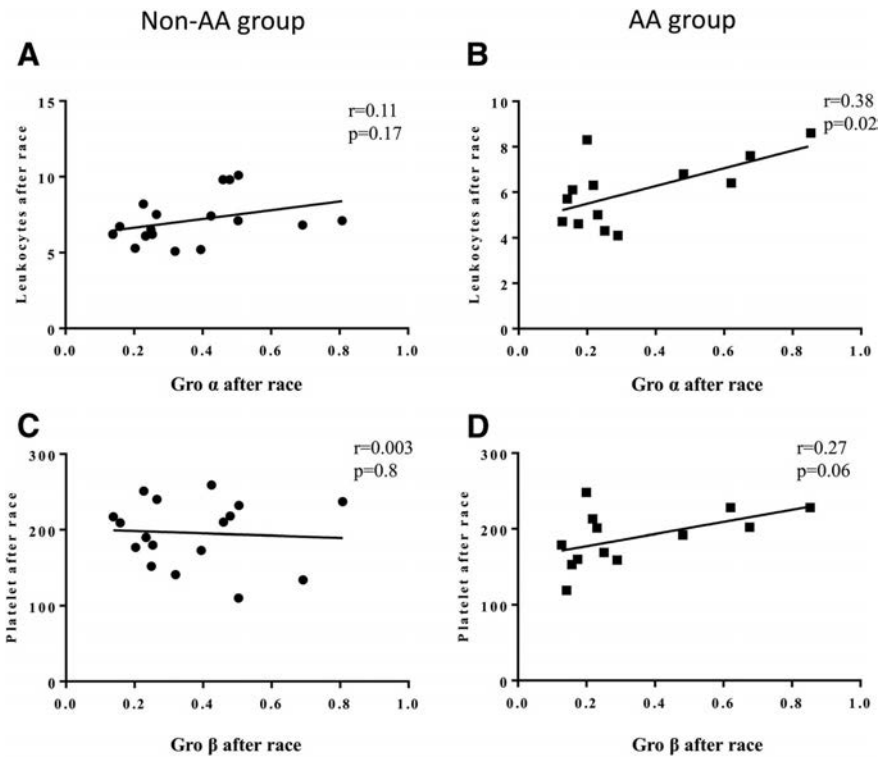

Fig. 4 Systemic correlations 48 h after the marathon (d2) between: salivary Gro-alpha levels with leukocyte counts in non-AA group (**a**) and AA group (**b**) and Gro-beta salivary levels and blood platelet counts in non-AA group (**c**) and AA group (**d**)

the immune system cannot be ruled out, and further studies in non-elite marathon runners should be performed to better clarify this important point.

Conclusion

In conclusion, our study documented significant changes in salivary and systemic immunological biomarkers in marathon runners after consuming a polysaccharide-based multi-ingredient supplement AA, before and after the race. Further research using randomized, double-blind, placebo-controlled design will extend knowledge of the potential benefits nutritional dietary supplementation with polysaccharides may have in marathon runners.

Abbreviations

AA: Standardized dietary plant-derived polydisperse polysaccharide Advanced Ambrotose© complex powder; BMI: Body mass index; CK: Serum creatine kinase; CRP: C-reactive protein; HGB: Hemoglobin; Hs-TnT: High-sensitivity troponin T; NT-proBNP: N-terminal pro-B-type natriuretic peptide; sIgA: Salivary secretory Immunoglobulin A

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors participated at the conception and design of the study. ER and LN were involved at data collection. All authors participated at data interpretation and analysis. ER, EC, OS and SV participated at manuscript drafting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

As discussed in the Participants section of the manuscript, this study was approved by an institutional review board. Protocol number: IIBSP-SUMMIT-2016-02. All participants provided written consent prior to their participation in the study.

Consent for publication

Not applicable.

Competing interests

The study was started prior to the corresponding author, Emma Roca joining the Global Scientific Advisory Board (GSAB)* at Mannatech, Inc. as related research to her PhD studies. The study was approved by the supervising Faculty/Professor and the University (Universitat Politècnica de Catalunya (UPC), Research Centre for Biomedical Engineering (CREB-UPC) and IRB without input from Mannatech, Inc.

Publisher's Note

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Chapter 5: Conclusions

The increasing popularity in such strenuous events such as a marathon can have consequences in several health parameters that have to be considered. The acute changes that happen, have to be understood and every runner has to act in kind.

The impact of a marathon in the healthy state of a non-elite runner is more serious than expected and either training hours, race time, recovery time, body weight, diet, and supplements, can significantly affect the performance and increase myocardial inflammation and strain, myocyte stress/injury, infection, body inflammation, acute kidney injury and muscle damage.

More specifically, analyzing blood and saliva biomarkers together with global bioimpedance measurements and nutritional intake in Barcelona marathoners pre-race, at the end and 48 h post-race, results in the different areas were obtained.

The following main conclusions have been drawn from the published articles and articles under revision included in this thesis:

Cardiovascular health

Cardiac biomarkers significantly increased during the marathon in all runners. This increase reached abnormal values for hs-TnT and ST2, and was significantly associated with worse running performance and less training hours. We don’t know if these elevated biomarkers values confer long-term increased risk in cardiovascular health (myocardial infarction, cardiovascular death, adverse ventricular remodeling, diastolic dysfunction, right ventricular dysfunction, among others) or are a cardiac sport-driven adaptation (Chapter 2).

In addition, the marathoners that consumed adequate amounts of unsaturated fat, potassium, and magnesium, performed better and presented better cardiovascular health (Appendix 3).

Immunity and infection

Analyzing all the marathoners before and after the race, changes in salivary antimicrobial proteins and chemokines were found. They were related to respiratory tract infections (upper and lower ones) and correlated with systemic defense cells, suggesting an important role of salivary immunity in strenuous exercise. Therefore, adequate exercise training and post-marathon recovery can be important to maintain a proper immunological profile and prevent the risk of developing a respiratory tract infection (Chapter 3).

Some types of supplements, like polysaccharide-based multi-ingredient supplements (glycan based), can have a positive effect on immune response after a marathon. Significant changes in salivary and systemic immunological biomarkers were found in those marathoners that took supplements. Thus, adequate supplementation before a marathon can bring benefits to an affected immune system after strenuous exercise (Chapter 4).

Renal health

There was a direct relationship between a marathon race in non-elite runners and serum biomarkers of kidney function and inflammation, with a high percentage of AKI stage 1 after finishing the race, but mostly recovered to baseline levels of serum creatinine and urea 48 h post-race. This transient reduction in renal filtration function was more related to inflammatory factors rather than muscle damage. Phase-sensitive measurements at 50 kHz with BIVA, could be a non-invasive method to assess kidney function in runners through the Xc/H 48 h post-race value with a decrease >3% with respect to the baseline value (Appendix 2).

Muscle damage and inflammation

The highest elevations of biomarkers associated with muscle damage and inflammation have occurred in those runners with the worst performance in a marathon. In addition, these runners have been the ones who have done less previous training. Some of the consequences of these biomarker elevations are an increased risk of injury and a longer recovery time (Appendix 3).

Nutrition

Marathoners with higher BMI values had worse performance and running economy in a marathon. An inappropriate weight to run a marathon involves problems beyond a poor performance. In addition, the consumption of mono- and poly-unsaturated fatty acids and correct intake of potassium and magnesium, result in a better performance (shorter race times) in marathoners, without compromising the health status assessed by a correct circulating lipid profile (Appendix 3).

Figure 40 shows a graphic summary of the results obtained from the articles published in this thesis (Chapter 2 to 4) and from the two articles submitted (Appendix 2 and 3).

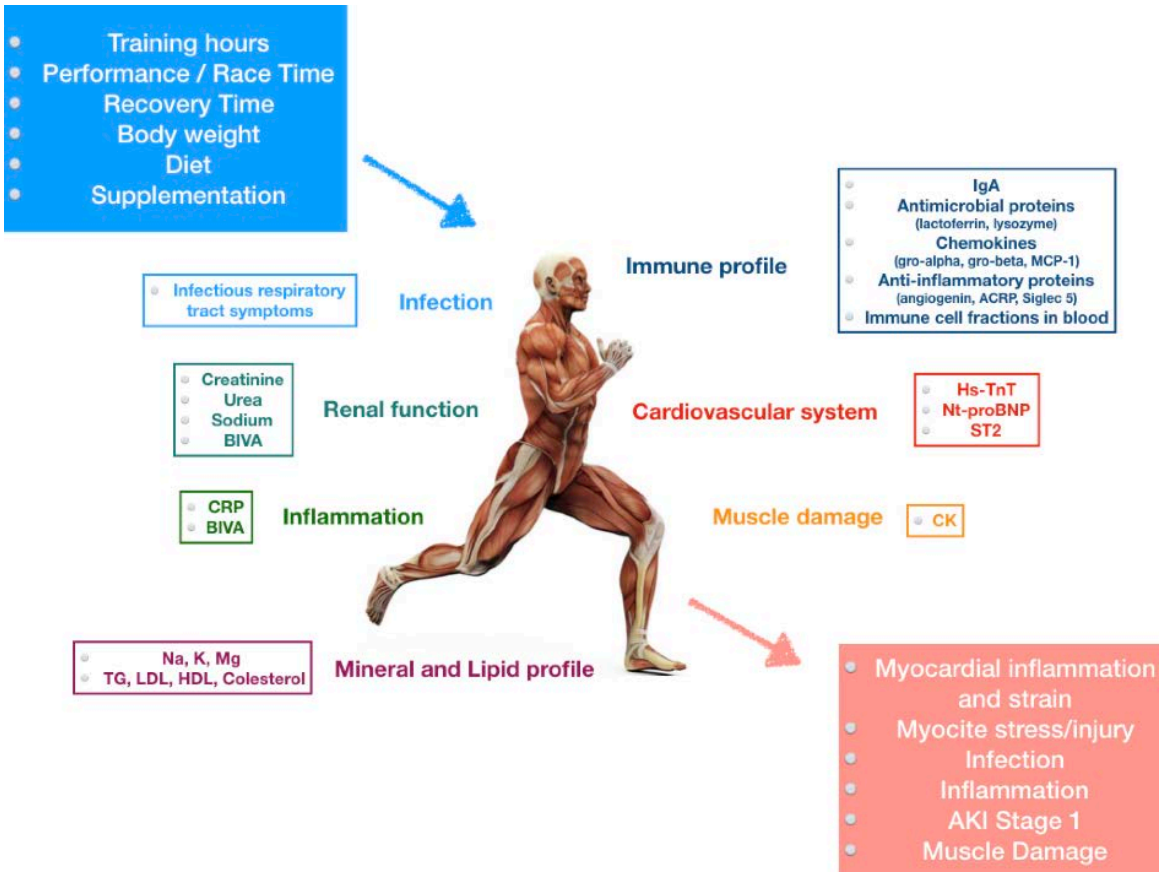

Figure 40. Graphic summary of the results obtained in this thesis.

Chapter 6: Perspectives

Despite Philippides, the Greek messenger who experienced sudden death after running more than 175 miles in 2 days, most of the ultra-distance racers have minimal risk running such distances, but scientific research can elucidate helpful data to avoid fatal consequences like Philippides.

More data and long-term follow-up studies in much more non-elite and elite athletes are needed. Also, the analysis of the race technique, specific muscle injury biomarkers, and monitoring at 7 or 14 days after a marathon or ultramarathon are needed to better understand the underlying mechanisms.

Concerning practical implications before a marathon, the non-elite runners should improve their personal performance through specific training. Localized bioimpedance (L-BIA) assessments and specific circulating biomarkers of muscle damage like CK-MM will be introduced in future marathons to evaluate muscle condition pre-race, immediately upon completion, and 48 h post-race.

In addition, diet and hydration guidelines for recreational runners need to be improved with certain macronutrients and electrolytes, in order to preserve healthy recreational runners. Related to supplement effect, further research using randomized, double-blind, placebo-controlled experimentation will extend knowledge of the potential benefits nutritional dietary supplementation with polysaccharides may have in marathon runners.

Thus, this thesis provides the first data related to the healthy state of amateur runners during a marathon; however more complementary, objective, and quantitative information about the impact of endurance running is needed. We must be able to provide data that allows us to make running an activity that is as healthy as possible, even in longer distances and over years of training and racing. After all what really kills us is a sedentary lifestyle, causing 6% of deaths worldwide.

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APPENDIX 1: Publications derived from this thesis

1. **Roca E**, Nescolarde L, Lupón J, Barallat J, Januzzi JL, Liu P, Cruz Pastor M, Bayes-Genis A. The dynamics of cardiovascular biomarkers in non-elite marathon runners. J Cardiovasc Transl Res 2017;10:206-8. http://doi.org/10.1007/s12265-017-9744-2
   IF: 2.337 / Q2 (Cardiac & Cardiovascular System)

2. Cantó E, **Roca E**, Perea L, Rodrigo-Troyano A, Suarez-Cuartin G, Giner J, Feliu A, Soria JM, Nescolarde L, Vidal S, Sibila O. Salivary immunity and lower respiratory tract infections in non-elite marathon runners. PloS One 2018;13(11): e0206059. http://doi.org/10.1371/journal.pone.0206059
   IF: 2.766 / Q1 (Multidisciplinary Science)

3. **Roca E**, Cantó E, Nescolarde L, Perea L, Bayes-Genis A, Sibila O, Vidal S. Effects of a polysaccharide-based multi-ingredient supplement on salivary immunity in non-elite marathon runners. Journal of the International Society of Sports Nutrition 2019;16(14):1-8. https://doi.org/10.1186/s12970-019-0281-z
   IF: 3.135 / Q1 (Sport Sciences)

Publications submitted, under review:

4. Nescolarde L, **Roca E**, Bogonez-Franco P, Hernández-Hermoso J, Bayes-Genis A, Ara J. “Relationship Between Bioimpedance Vector Displacement and Renal Function After a Marathon in Non-Elite Runners” to be considered for publication in Frontiers in Physiology, section Exercise Physiology. Manuscript submission - 505424
   IF:3.394 / Q1 (Physiology)

5. **Roca E**, Nescolarde L, Brotons D, Bayes-Genis A, Roche E. “Macronutrient and mineral intake effect on racing time and cardiovascular health in non-elite marathon runners”. Nutrition. Manuscript submission
   IF:3.734 / Q1 (Nutrition & Dietetics)

International conferences:

1. **Roca E**, Nescolarde L, Lupón J, Barallat J, Pastor C, Bayes-Genis A (2017): *The Dynamics of Cardiac Biomarkers in Amateur Marathon Runners*. **4th World Congress on Acute Heart Failure (28apr-2may 2017), Paris, France.**

2. **Roca E**, Nescolarde L, Perera A, Bogónez-Franco P, Soria JM, Sibila O, Vidal S, Canto E, Roche E, Brotons D, Bayes-Genis A (2016): *Gene expression, biochemical markers and physiological parameters to evaluate the military adaptation to the long and hard exertion. Predict the performance for the stressful and exigent military exercises.* **Defense Innovation technology acceleration challenges (29nov-1dec 2016), Austin, Texas**.

National conferences:

1. **Roca E**, Nescolarde L, Lupón J, Barallat J, Pastor C, Bayes-Genis A (2017): *Dinámica de diferentes biomarcadores cardíacos en maratonianos amateurs.* **Congreso de las Enfermedades Cardiovasculares, SEC 2017 Madrid, España.**

2. **Roca E**, Nescolarde L, Lupón J, Barallat J, Pastor C, Bayes-Genis A (2017): *Dinàmica de diferents biomarcadors cardíacs en maratonians amateurs*. **29è Congrés de la Societat Catalana de Cardiologia (25-26 maig 2017), Barcelona, España.**

APPENDIX 2

Relationship Between Bioimpedance Vector Displacement and Renal Function After a Marathon in Non-Elite Runners (505424)

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ABSTRACT

PURPOSE: This study investigates the relationship between whole-body bioimpedance vector displacement, using bioelectrical impedance vector analysis and renal function, through serum biomarkers (creatinine, urea, sodium, C-reactive protein, creatine kinase) and urine biomarkers after a marathon.

METHODS: Nineteen non-elite runners were measured 24 h pre-race, immediately post-race and 48 h post-race. The bioimpedance measurements were analysed by BIVA using the Hotelling's T2 test. Runners were divided according to a cut-off of serum creatinine level immediately post-race in G1 (<1.2 mg/dL of serum creatinine level) and G2 (≥1.2 mg/dL of serum creatinine level). The increase of serum creatinine levels in 83% of G2 runners was related to acute kidney injury (AKI) stage 1.

RESULTS: Neither G1 nor G2 showed a creatinine clearance rate (CCr) lower than 60 mL/min. G2 showed a significant increase in CRP values 48 h post-race compared to baseline than G1 ($P<0.05$) over 5 mg/L (6.8 ~ 15.2) in 92% of the runners and CK values over 215 U/L (282 ~ 1882) 48 h post-race in 100% of the runners. By BIVA, the 95% confidence ellipses of G2 showed shorter bioimpedance vectors than G1 with a noticeable minor Xc/H ($P < 0.01$), indicating an expansion on extracellular water and inflammation. The runners with Xc/H 48 h post-race values ≤ 30.5Ω with a decrease from -3% to -12% respect to Xc/H value 24 h pre-race, indicated AKI stage 1 with 85.7% sensitivity and 91.7% specificity, with a direct correlation between AKI stage 1 with greater CRP values 48 h post-race and bioimpedance vector displacement but not with CK values 48 h post-race.

CONCLUSION: In non-elite runners, with transient AKI stage 1, a follow-up of kidney function by serum biomarkers along with the BIVA can avoid noticeable renal functional impairments in future races.

Keywords: marathon race, BIVA, serum creatinine, C-reactive protein, serum creatine kinase, AKI stage 1.

APPENDIX 3

Macronutrient and mineral intake effect on racing time and cardiovascular health in non-elite marathon runners (NUT-D-19-01000)

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ABSTRACT

Objective: The aim of this study was to analyze, in recreational marathon runners, the intake of specific macronutrients and minerals that could influence cardiovascular health.

Methods: 37 males were grouped in two groups according to their 50% percentile race time (3.39 h), dividing into fast (G1: 3.18 ± 0.18 h) and slow runners (G2: 3.84 ± 0.42). Anthropometric parameters, macronutrients and mineral records were collected before the race. Minerals (Na+, K+ and Mg2+), lipid profile (triglycerides, LDL, HDL and cholesterol), inflammation (C-reactive protein), muscle damage (creatine kinase) and cardiovascular health (high-sensitive troponin-T, N-terminal pro-B-type natriuretic peptide and ST2) were analyzed in blood 24h before, immediately after, and 48h post-race.

Results: Weight (G1: 72.60 ± 7.38 kg, G2: 76.92 ± 6.51 kg; $p < 0.05$) and body mass index (G1: 22.34 ± 1.64 kg/m2, G2: 24.50 ± 1.99 kg/m2; $p < 0.01$) were significantly different between the groups. Moreover, G1 consumed significantly ($p < 0.01$) more mono- and poly-unsaturated fatty acids than G2, and presented higher iron, potassium, and magnesium intake. Regarding blood lipid profile, G2 presented significantly higher triglyceride values and lower levels of high-density lipoprotein ($p < 0.01$). The Hs-TnT marker of myocyte stress/injury was significantly higher ($p < 0.05$) in G2 reaching values above 250 ng/L, and 81% of the runners (30 from 37) presented above cut-off ≥ 14 ng/L post-race values.

Conclusions: Marathon runners consuming adequate amounts of unsaturated fat, potassium and magnesium, performed better and presented better cardiovascular health.

Keywords: macronutrients; minerals; marathoners; performance; cardiovascular health.

APPENDIX 4: Publications derived from data acquired before this thesis

Publications indexed:

1. Carmona G, **Roca E**, Guerrero M, Cusso R, Cadefau JA (2018): Fibre-Type-Specific and Mitochondrial Biomarkers of Muscle Damage after Mountain Races. Int J Sports Med 39:1-10. https://doi.org/10.1055/a-0808-4692

2. Vallverdú M, Ruiz-Muñoz A, **Roca E**, Caminal P, Rodriguez F, Irurtia A, Perera A (2017): Assessment of Heart Rate Variability during an Endurance Mountain Trail Race by Multi-Scale Entropy Analysis. Entropy 19, 658:1–17. https://doi.org/10.3390/e19120658

3. Maqueda M, **Roca E**, Brotons D, Soria JM, Perera A (2017): Affected Pathways and Transcriptional Regulators in Gene Expression Response to an Ultra-Marathon Trail: Global and Independent Activity Approaches. PLoS ONE 12 (10):e0180322. https://doi.org/10.1371/journal.pone.0180322

4. Messier F, Le Moyec L, Santi C, Gaston AF, Triba M, **Roca E**, Durand F (2017): The impact of moderate altitude on exercise metabolism in recreational sportsmen: a nuclear magnetic resonance metabolomic approach. Appl Physiol Nutr Metab 42(11):1135-1141. https://doi.org/10.1139/apnm-2016-0717

5. Faoro V, Deboeck G, Vicenzi M, Gaston AF, Simaga B, Doucende G, Hapkova I, **Roca E**, Subirats E, Durand F, Naeije R (2017): Pulmonary vascular function and aerobic exercise capacity at moderate altitude. Med Sci Sports Exerc 49 (10):2131-2138. https://doi.org/10.1249/MSS.0000000000001320.

6. Sanz de la Garza M, Grazioli G, Bijnens BH, Pajuelo C, Brotons D, Subirats E, **Roca E**, Sitges M (2016): Inter-individual variability in right ventricle adaptation after an endurance race. Eur J Prev Cardiol 23(10):1114–1124. https://doi.org/10.1177/2047487315622298

7. Sanz de la Garza M, Grazioli G, Bijnens BH, Sarvari SI, Guasch E, Pajuelo C, Brotons D, Subirats E, Brugada R, **Roca E**, Sitges M (2016): Acute, Exercise Dose-Dependent Impairment in Atrial Performance During an Endurance Race:

2D Ultrasound Speckle-Tracking Strain Analysis. JACC: Cardiovascular Imaging, 9(12):1380–1388. http://doi.org/10.1016/j.jcmg.2016.03.016

8. Gaston AF, **Roca E**, Doucende G, Hapkova I, Subirats E, Durand F (2016): Réponses physiologiques à l'exercice en altitude modérée : Intérêt de la mesure de la SpO2. Science & Sports 31(1): 6–12. https:// doi.org/10.1016/ j.scispo.2015.06.006

9. Carmona G, **Roca E**, Guerrero M, Cussó R, Irurtia A, Nescolarde L, Brotons D, Bedini JR, Cadefau JA (2015): Sarcomere Disruptions of Slow Fiber Resulting From Mountain Ultramarathon. Int J Sports Physiol & Performance 10:1041–1047. https://doi.org/10.1123/ijspp.2014-0267

International conferences:

1. Caixal G, Grazioli G, Guasch E, Sanz M, Mont L, Subirats E, Brotons D, Brugada R, **Roca E**, Sitges M (2014): Ventricular repolarization changes after a cross.running race: a substrate for arrhythmic events?. SEC Octubre 2014, Santiago Compostela.

2. Sanz de la Garza M, Grazioli G, Bijnens B, Pajuelo C, Guasch E, Subirats E, Brotons D, Brugada R, **Roca E**, Sitges M (2014): High interindividual variability in the acute dose-response of the right ventricular performance in crossrunning participants. Europrevent, Amsterdam.

3. Sanz de la Garza M, Grazioli G, Bijnens B, Pajuelo C, Guasch E, Subirats E, Brotons D, Brugada R, **Roca E**, Sitges M (2014): How does the right ventricle adapt to high intensity endurance exercise? Insights from segmental myocardial deformation imaging. Europrevent, Amsterdam.

4. Melia U, Vallverdu M, **Roca E**, Brotons D, Irurtia A, Cadefau JA, Caminal P, Perera A (2014):Heart Rate Variability in Ultra-Trail Runners. Computing in Cadiology, Boston 2014; 41:997-1000.

5. Deboeck G, Vicenzi M, Simaga B, Naeije R, Gaston A, Hapkova I, Doucende G, **Roca E**, Subirats E, Durand F, Faoro V (2014): Pulmonary vascular function during exercise at sea level and moderate altitude in normal subject. American Thoracic Society 2014 International Conference, May 16-21, 2014 - San Diego.

6. Faoro V, Vicenzi M, Simaga B, Naeije R, Gaston A, Hapkova I, Doucende G, **Roca E**, Subirats E, Durand F, Deboeck G (2014): Fonction vasculaire pulmonaire à l'effort chez les sujets sains en moyenne altitude. 7e Forum Européen Coeur, Exercice et Prévention, 20-22 Marzo Paris.

National conferences:

1. Lopez A, Maqueda M, **Roca E**, Perera A (2016): Desarrollo de una plataforma web para el acceso interactivo a una base de datos SQL con información biológica de competiciones deportivas. XXXIV Congreso Anual de la Sociedad Española de Ingeniería Biomédica (CASEIB 2016).

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APPENDIX 5: Races/events analyzed 2012-19

		Races or events analyzed	km	men	elite men	women	elite women	Total
Pre thesis period	2012	Cavalls del Vent (Berguedà, Catalunya)	84	9	4	2	3	18
	2013	Volta Cerdanya Ultra Fons (Cerdanya) (pre/post)	14	17	2	8	0	27
		Volta Cerdanya Ultra Fons (Cerdanya) (pre/post/ 24/48)	35	25	5	17	3	50
		Volta Cerdanya Ultra Fons (Cerdanya) (pre/post/ 24/48)	55	17	0	17	0	34
		Moderate altitude study with STAPS-Perpignan Univ	-	20				20
	2014	Ultra Trail Barcelona (pre/post/24)	100	1	1	0	1	3
		Ultra Trail Barcelona (pre/post/24)	69	6	0	0	0	6
		Ultra Trail Barcelona (pre/post/24)	42	1	0	0	0	1
		Transvulcania (Canary Islands) (pre/post/24)	74	8	0	0	1	9
		Zegama (Basque Country) (pre/post)	42	11	0	0	1	12
		Volta Cerdanya Ultra Fons (Cerdanya) (pre/post)	88	10	0	2	0	12
		Volta Cerdanya Ultra Fons (Cerdanya) (pre/post)	35	11	0	0	1	12
		Transalpine (Germany, Italy) (pre/post)	293	7	1	7	1	16
	2015	Trail Menorca (pre/post/24)	100	7	0	0	0	7
		Trail Menorca (pre/post)	185	9	2	0	0	11
Thesis period	2016	Metabolomic study with STAPS-Perpignan Univ	-	24				24
		Barcelona marathon (runners)	42	57		24		81
	2017	Barcelona marathon (runners)	42	29		6		35
		Barcelona marathon (no racing this year)	-	12		9		21
		Altitude & perfomance study with STAPS-Perpignan Univ	-	30				30
		Sherpa Everest altitude study with Ferran Latorre	-	40		3		43
		Ultra Trail Pirineus (Berguedà)	45	16	7	2		25
		Marnaton (Barcelona, Sant Feliu and Begur)		5		3		8
	2018	Barcelona marathon (runners)	42	23		5		28
		Barcelona marathon (no racing this year)	-	13		4		17
		K2 altitude study with Alex Txikon	-	12		4		16
		Ultra Trail Pirineus (Berguedà)	45	38		6		44
		Ultra Trail Naut Aran (Vall d'Aran)	54	11		1		12
	2019	Barcelona marathon (runners)	42	26		4		30
		Barcelona marathon (no racing this year)	-	14		8		22
TOTAL		**30**		**509**	**22**	**132**	**11**	**674**

APPENDIX 6: Races done as an endurance athlete

Year	Pos.	Race	km
2019	1	Trail Vall de Ribes (2 stages)	65
	3	Ultra Trail Australia	100
2018	2	Run Rabbit Run, Colorado	170
	7	Ultra Trail Lavaredo (Ultra Trail World Cup) (1st veteran)	110
	2	Ultra Trail Penyagolosa (Ultra Trail World Cup)	110
	1	Costa Brava Stage Run (3 stages)	85
2017	2	Run Rabbit Run, Colorado	170
	23	Comrades marathon, South Africa	89
	1	Half marathon Coll de Pal	20
	1	Half marathon Tossa de Mar	21
	2	Half marathon Montornès del Vallès	21
2016	3	Diagonale des Fous (Ultra Trail World Cup) in Reunion Island	170
	2	Hardrock, Colorado, USA (9th overall)	160
	1	Emmona mountain marathon	42
	1	Romanic extrem mountain marathon	42
	1	Estels del Sud ultra marathon	65
2015	1	TCN Camí de Cavalls, Menorca	33
	5	Western States, California, USA	160
	1	Run Rabbit Run, Colorado, USA	166
	1	Otter Trail Run, South Africa	42
2014	1	Transalpine Run, Germany, Italy (8 stages)	293
	1	Leadvile, Colorado, USA (8th overall)	161
	1	Cruce de los Andes, Chile-Argentina (3 stages)	120
	1	K42 Mallorca	47
	1	Ultra Trail Barcelona	100
	4	Transvulcania, Canary Islands	74
2013	2	Cruce de los Andes, Chile-Argentina (3 stages)	120
	1	K42 Mallorca	42
	1	Magaluf Marathon, Mallorca	42
	1	Ultra Trail Barcelona	114
	2	Kilian's Classic, Font Romeu, France	45
	4	Speedgoat, Utah, USA	50
	3	Ultra Trail Mont Blanc, Chamonix, France (21th overall)	169
	1	Collserola Marathon, Barcelona	43
	1	K42 Tenerife	44
2012	dsf	TransGranCanaria, Canary Islands	123
	1	Highland Fling Race, Scottish Ultra Trail Champs, Scotland	85
	1	Q50 Madrid	50
	1	Volta Cerdanya	35
2012	3	Ultra Trail Mont Blanc, Chamonix, France (37th overall)	110
	4	Ultramarathon Cavalls del Vent, Bagà	85
2011	2	Marathon des Sables, Morocco (23th overall)	380
	3	Ultramarathon Cavalls del Vent, Bagà	90
	2	Triatlhon sprint La Molina, Cerdanya	5
	3	Verticalp La Molina, Cerdanya	15
2010	9	Ironman Nice, 1st age group, classified to Hawaii	42
	10	Zarautz Triathlon, Basque Country (Half Ironman distance)	21
	6	Half Challenge Maresme (half Ironman distance)	21
	2	B-distance triathlon, Banyoles, Pla de l'Estany	21
	1	Winter Triathlon Championship, Tuixén, Alt Urgell	10
	4	Winter Triathlon Spanish Championship, Ansó, Huesca	10
	1	Mountain Duathlon Championship, Folgueroles, Osona	10
	1	Mountain Duathlon St. Joan of Abadesses, Ripollès	10
	1	Road Duathlon Castellfollit de la Roca, Garrotxa	10
	2	Road Duathlon Catalonia Championship, St. Joan Ab., Ripollès	10
	2	Road Duathlon Vic, Osona	10
2009	1	Triathlon Puigcerdà, Cerdanya	10
	1	Half mountain Marathon Vic, Osona	22
	12	Maresme Challenge (Ironman distance with a time of 10h30min)	42
2004	1	Half Marathon La Molina, Cerdanya	25
	1	Mountain Duathlon Puigmal	10
2002	4	SkySki Trophées Mont-Blanc.	30
	3	World Cup mountain marathon, Kima, Italy	65
2001	1	European Championship Mountain Marathon, Viella, Vall d'Aran	42
	2	Mountain Marathon, Madrid	42
	1	Mountain race, Molina, Cerdanya	25
	1	Catalonia Championship mountain Duathlon, Mataró, Barcelona	10
2000	7	Championships mountain Marathon, Cervinia, Italy	42
	2	Mountain Marathon, Kima, Val Masino, Italy	65
	1	Catalonia Championship mountain Duathlon, Cerdanyola, Barcelona	10
	5	Spanish Championship Winter Triathlon, Reinosa, Cantàbria	10
1999	2	Spanish Championships Duathlon, Córdoba	10
	2	X-Treme Aneto Nike Marathon, Benasque, Huesca	42
1998	1	Catalonia Championships mountain Duathlon	10
	3	Catalonia Championships Duathlon, Reus, Tarragona	10
	1	X-Treme Aneto Nike Marathon, Benasque, Huesca	42
	1	Spanish Championships long distance Duathlon	22
TOTAL Km			5044